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13. ABSTRACT (Maximum 200 Words) Loss of heterozygosity in the 1p31 region is a common event in breast cancer development suggesting the location of a tumor suppressor gene. Using a combination of molecular cloning and bioinformatics strategies, we identified 18 known genes and 16 predicted genes within the critical region of 1p31. Molecular analysis of selected genes such as TTC4 and the progesterone receptor (PTGFR) did not identify nonsense mutations in breast tumors. A comparison of expression profiles for the 18 known genes between tumor and normal tissue demonstrated seven which were down regulated in breast cancer. No nonsense mutations were found in these genes although protein altering missense mutations were detected specifically in tumor DNA compared with the paired normal tissue. Verification of the role of any of these genes in breast cancer will require functional assays. During the course of these analyses, we also identified the CLCA2 gene in 1p31 which was down regulated in 50% of breast cancer and cell lines. Bisulfite sequencing showed that promoter methylation was the cause of the inactivation of this gene and treatment with 5-azacytidine could reactivate it in the MDA-MB435 and MDA-MB231 cell lines. These results strongly suggest that CLCA2 could be a critical gene in 1p31 for breast cancer development.						14. SUBJECT TERMS Breast cancer	
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	13
Reportable Outcomes.....	14
Conclusions.....	14
References.....	n/a
Appendices.....	16

ISOLATION OF GENES FROM CHROMOSOME REGION 1p31 INVOLVED IN THE DEVELOPMENT OF BREAST CANCER

INTRODUCTION

One of the hallmarks of tumor suppressor genes is that both copies must be inactivated for the development of the malignant phenotype. A phenomenon associated with this inactivation is that a single recessive mutation is exposed following loss of a chromosomal region containing the normal copy of gene. This event is usually manifested in tumors as loss of heterozygosity (LOH) when compared to normal tissue from the same individual. Genome screening for LOH in breast cancer has identified chromosome region 1p31 as one commonly (50%) involved in LOH indicating the site of a tumor suppressor gene. By comparison of LOH in a large number of tumors the minimal region has been defined by two markers which are 500-800 Kb apart. An extended region of loss spans 2-3 Mbp. The overall aim of this project was to characterize the region of chromosome 1p31 which shows consistent loss of heterozygosity in breast tumors by constructing a BAC contig across the region, searching for genes in this contig and then performing a mutation analysis to determine which ones are involved in tumorigenesis.

BODY

We had previously established a YAC contig across the critical 1p31 region and used this collection of large genomic clones as a mapping tool for newly identified genes in the region. One of these genes contained the EST marker 16a09, which identified a 2.0 Kb transcript on Northern blots. Using 5' RACE we eventually generated the full length sequence of this gene which is 2005 bp long. Conceptual translation identified a tetratricopeptide repeat motif at the C-terminal end of this gene and a coiled coil motif at the N-terminal end of the reading frame. It has been shown that genes with these motifs are involved in protein-protein binding. The 16a09 gene was therefore a novel member of this family and is the fourth member of the human gene family. In consultation with the Human Gene Nomenclature Committee this gene was named TTC4. Expression analysis showed that TTC4 is expressed almost in all tissues including normal breast (Su et al 1999). To look for mutations we performed a preliminary mutation analysis in 8 breast tumor cell lines by using RT-PCR followed by an SSCP analysis of the PCR products. We were unable to identify band shifts in any of these cell lines indicating that mutant transcripts were not present. It was possible, however, that mutant RNAs would be unstable and so would not be detected in these types of analyses. In order to make a more detailed mutation analysis, the exon/intron structure of the gene was established from the DNA sequence of a BAC containing the TTC4 gene. It was shown that TTC4 contains 10 exons which are spread over 25 Kb of genomic DNA. Knowing the exon/intron boundaries made it possible for us to construct PCR primer pairs which could then be used to amplify each of the individual exons from genomic DNA for SSCP analysis. Using this approach we were still unable to detect any mutations in the coding region for this gene, although polymorphisms outside the coding region were readily identified (Su et al 1999). Although it is still possible that modifications in the promotor of this gene is, in some way, connected to an altered expression pattern in breast cancers, this would not be consistent with the observation of LOH which is generally thought to result in the exposure of a mutation in a recessive oncogene. Thus we feel that we have proven that TTC4 is not the target in the LOH region in 1p31. After the considerable effort expended on the analysis, of what was at first sight an excellent candidate gene, we returned to completing the BAC contig across the region. This effort ran throughout the course of this project and was accomplished from a combination of BAC 'walking' and the completion of the human genome sequence database. The move by the PI to Roswell Park Cancer Institute in late 2000 made all of the clones from this region available since this was where the BAC libraries for the genome project were generated and are maintained.

As part of this mapping effort we determined that the progesterone receptor gene (PTGFR) was located at the distal border of the LOH region. Because of the potential involvement of this receptor in breast cancer development, we decided to employ an SSCP/mutation screening and DNA sequencing. In order to establish the exon/intron structure, we identified a BAC clone which contained the PTGFR gene and then, by aligning the

cDNA sequence with the genomic sequence, were easily able to establish the exon structure. The PR gene only contains three exons and so primers were designed to amplify the individual exons and subject them to SSCP analysis. This approach identifies single strand PCR products which show altered mobility on acrylamide gels, indicative of a DNA sequence change (either mutation or polymorphism). Since each of the PTGFR exons were large (>250 bp), we designed a series of overlapping PCR products to cover each exon. We used 32 tumor samples from breast cancers in this analysis and identified a number of band changes. When sequenced, these changes proved to be silent polymorphic variants resulting in silent changes in the amino acid code for the gene. From this survey, therefore, we concluded that the PR gene is not frequently mutated in breast cancer (Sossey-Alaoui et al 2001).

Analysis of the draft sequence of the human genome from the public database (<http://www.ncbi.nlm.nih.gov>) showed that the common region of LOH in breast cancer at the 1p31 region encompasses 2 BAC contigs (Proximal-NT_037488-NT_004483-Distal). This region is defined by STS markers D1S207 and D1S2876, which map to contig NT_037488 and NT_004483, respectively. The genomic sequence between these two markers is estimated to be 3.5-Mbp long. Since a preliminary annotation of the sequence from these two BAC contigs was available to us in the NCBI public database, it was possible to determine their gene content. 18 known genes (Figure 1) and 16 predicted genes (Table 1), with similarities to genes of known function map to this region.

Figure 1: List of known genes that map to the 1p31 breast cancer critical region.

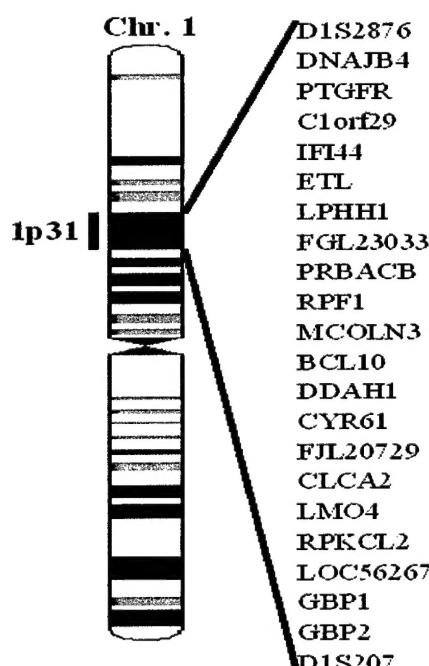


Table 1: List of predicted genes that map to the 1p31 breast cancer critical region.

Gene Symbol	1.0 FULL NAME
LOC204993	Similar to Nuclear protein SkiP
LOC149050	Similar to Phosphoserine Aminotransferase, Isoform 2
LOC163813	NA
LOC163812	Similar to Glutathione-Dependent Formaldehyde Dehydrogenase
LOC253020	Similar to RIKEN cDNA 1200009K13
LOC127507	Similar to Ribonucleoprotein A1
LOC127509	NA
LOC163814	NA
LOC126718	Similar to Ribosomal Protein L7
LOC163816	Similar to Ribosomal Protein L10a
LOC204952	NA
LOC204953	NA
LOC204954	NA
LOC253208	NA
LOC253209	NA
LOC149052	Putative Tumor Suppressor ST13

Simply because these genes map to the breast cancer critical region makes them equal candidates for the 1p31 breast tumor suppressor gene. To implicate any of these genes in breast tumorigenesis, however, it was

necessary to determine whether these genes are expressed in the normal breast epithelium, and thereafter, undertake mutation analysis in a panel of breast tumors.

The complete sequence of the cDNA of each gene has been deposited in the public databases (<http://www.ncbi.nlm.nih.gov/>). In order to conduct a mutational study that covers the whole coding sequence as well as the splice junctions and the promoter region of candidate genes, it was necessary to establish the genomic structure and determine the exon-intron boundaries of each one of these genes, which has, therefore, allowed an exon-by-exon mutational analysis (see below). A priority was given to the genes that are expressed in normal human breast epithelium. Therefore, it was also necessary to establish the expression profile for all the known genes as well as the predicted ones.

EXPRESSION ANALYSIS OF THE BREAST CANCER CANDIDATE GENES IN BREAST TUMORS AND CELL LINES.

We have used RT-PCR analysis to monitor the expression of known genes that map to breast cancer critical region on 1p31. From this list of genes (Figure 1 and Table 1), it appeared clearly that half (8 out of 16) of the predicted genes (LOC genes) have no similarity to known genes. Therefore, it was necessary to establish the authenticity of these genes by analyzing their expression profile using RT-PCR and Northern blot analyses. We also determined whether these genes have similarity to known or predicted genes from the data available from the mouse gene map, which is another indication of their authenticity. On the other hand, expression analysis of several of the known genes in breast cancer cell lines and breast tumors (Figure 2) has already identified at least 8 potential candidate genes which showed differential expression patterns (ETL, DNAJB4, MCLOLN3, IFI44, CYR61 BCL10, DDAH1 and FJL20729). At the same time, this analysis has helped assign low priority to several genes, which are ubiquitously expressed in the cell lines and tumors (GBP1, GBP2, LOM4 ...etc).

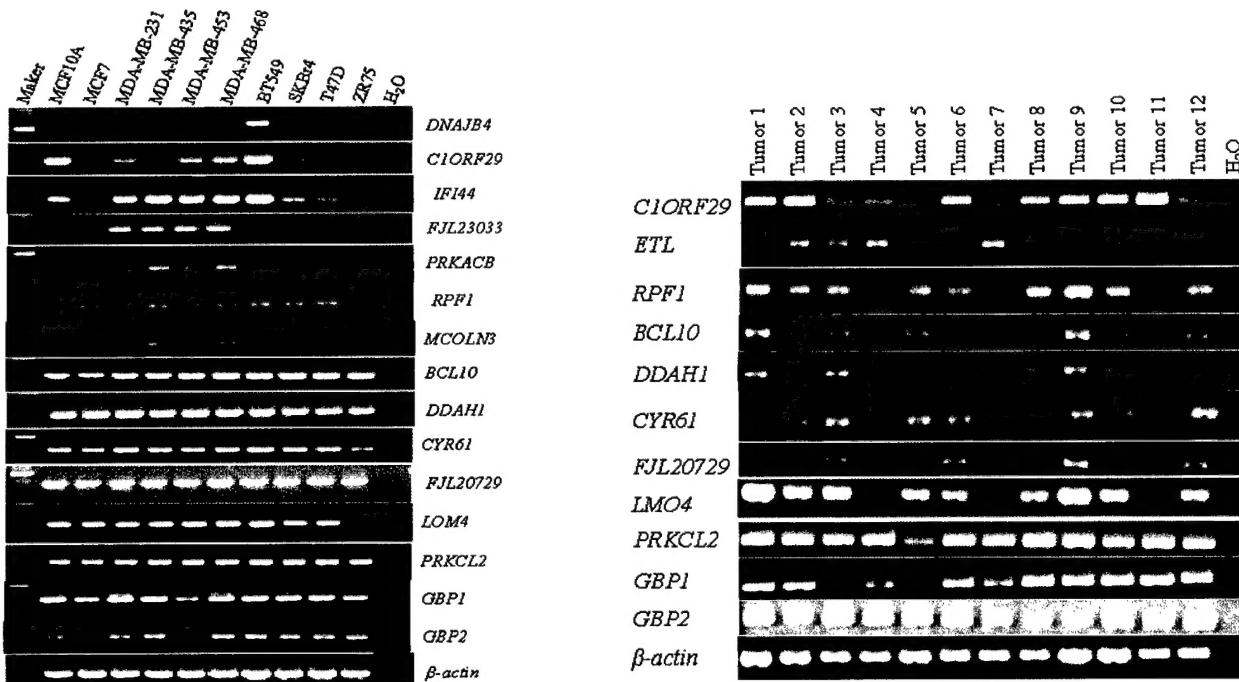


Figure 2: Expression analysis of the known genes from the 1p31 breast cancer critical region in breast cancer cell lines (left panel), and in breast tumors (right panel).

MUTATION ANALYSIS IN BREAST TUMORS.

The common region of LOH in breast tumors at the 1p31 locus is about 3.5-Mbp long and contains more than 30 known and predicted genes. Even after individual genes have been identified the problem is still to determine which one is responsible for breast cancer pathogenesis. LOH strongly suggests that loss of function of a critical gene is important in tumorigenesis. LOH can result either from heterozygous deletions of the critical gene leaving only the mutant gene in the cell, or the generation of homozygosity as a result of non-disjunction for the chromosome carrying the specific mutation. In this case the mutation is homozygous in the tumor cells. In either case the remaining gene in these tumors should carry subtle inactivating changes such as; point mutations, splicing errors, and microdeletions and insertions which result in frameshifts.

In the past, we used the single strand conformation polymorphism (SSCP) technique to prescreen exons of genes before sequencing. Although it is clearly more time-efficient and cheaper to adopt a prescreening approach (rather than a wholesale sequencing of exons at the outset), one of the criticisms of SSCP is that it is only possible to identify up to 70% of mutations (depending on the technical expertise of the laboratory). In fact, this is not such a problem when trying to implicate a gene in a particular disease, since a reasonable number of mutations alone would be proof of its involvement. With the development of denaturing high performance liquid chromatography (DHPLC) approaches, however, the efficiency of DNA mutation detection rises to over 95% and has the added advantage of being automated, quick and does not require the use of radioactivity or gels. We have therefore routinely adopted this approach using the Transgenomics WAVE instrument. The WAVE machine uses a DHPLC format with a proprietary resin for the separation of DNA. The passage of DNA through the column is monitored using UV absorbance. The principal is, that heteroduplexes of different sequences melt at lower temperatures and so pass through the column earlier than the homoduplexes. This system uses PCR products which are generated using specifically designed primers using the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Individual exons are initially amplified from a standard DNA sample (Clontech) using 8:1 mixture of Taq:Pfu polymerases. The proofreading capabilities of Pfu minimizes errors due to misincorporation of bases during PCR amplification. These PCR products are then used to establish the appropriate melting profiles for each exon (see Figure 3). The sequence of the amplified fragment is first analyzed with a DHPLC melting profile software program, to establish the range over which the optimal denaturation of the PCR product would occur. The sample is then denatured and reannealed using the following parameters: 95°C for 5 minutes; 70-degree slope down to 25°C at a rate of 1.5 per minute; 4°C hold. For the initial melting profile 5 µl of sample are run through the column for each of 3 different temperatures as determined by the software. The elution profile is viewed as a graph of absorbance over time and the temperature that gives the sharpest single peak at an elution time between 5 and 6 minutes is chosen. When the appropriate melting temperature for the PCR product has been established the PCR reaction is carried out using DNA from the tumor cells. Prior to running these samples on the WAVE they are mixed 6:4 with normal DNA and denatured and cooled. At this point homoduplexes and, if there is any sequence change, heteroduplexes are formed. The appearance of any early eluting peak signifies the presence of DNA sequence differences (see Figure 3). The DHPLC offers the advantage of automation on 96 well format which allows much higher throughput than normal gel approaches. Samples showing heteroduplexes can be sequenced to establish the nature of the sequence variation. In studies of a series of genes located within regions of the genome thought to harbor tumor suppressor genes, we have shown the effectiveness of this analysis and have clearly identified single base changes (mostly polymorphisms) in genes under study.

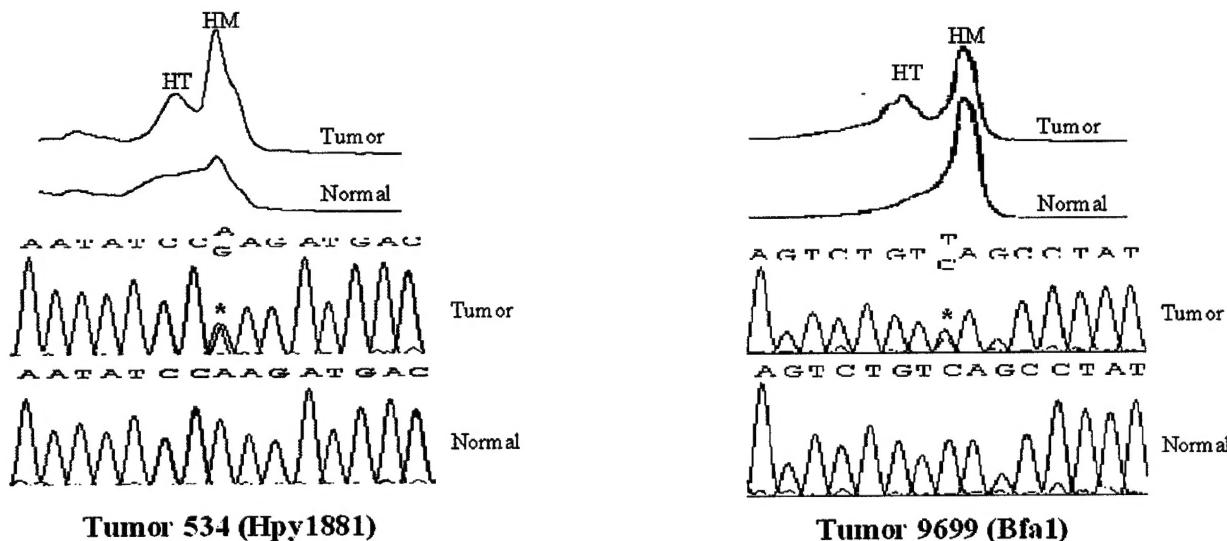


Figure 3: WAVE™-DHPLC mutation analysis of CLCA2 in breast tumour 534 (left) and tumour 9699 (right). In the absence of mutations the two alleles of CLCA2 form a homoduplex (HM) which elutes from the column at the same point. Any mismatch due to mutations leads to the formation of a heteroduplex (HT) which elutes earlier. Only homoduplexes are detected in the normal DNAs, but the corresponding tumour DNAs show heteroduplexes. When the DNAs showing the heteroduplexes were sequenced the presence of a heterozygous base change was seen (double peak at *). Tumour 534 has an A 2435 G substitution in exon 14 (Gln812Arg), and tumour 9699 has a G 976 A substitution in exon 7 (Asp326Asn). The sequence of the opposite strand of exon 7 is shown. The restriction enzymes that were used to verify the authenticity of the mutations are given in parenthesis.

A total of 58 breast tumors and 9 breast cancer cell lines were analyzed for point mutations in the coding sequence and splice sites of 7 candidate genes from the 1p31 critical region. The results of this mutation screening is summarized in Table 2. From this analysis we identified a number of coding region mutations in various genes but no stop codon mutations normally seen in tumor suppressor genes.

It is still possible that these point mutations affect protein function but this is not predictable from the sequence change alone. To examine this possibility, we would need to undertake functional studies which were beyond the scope of this analysis. The alternative was that gene expression was affected by promoter inactivation.

METHYLATION ANALYSIS IN BREAST CANCER CELL LINES

In higher eukaryotes, DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide. This modification has important regulatory effects on gene expression, especially when CpG-rich regions, located in the promoter regions of many genes are involved. Aberrant methylation of the normally unmethylated CpG islands has been documented as a relatively frequent event in immortalized and transformed cells and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers. In this case, instead of coding region mutations, promoter region hypermethylation provides an alternative means of eliminating tumor suppressor gene function. Analysing the methylation status of CpG islands of genes from the 1p31 region, therefore, is an important strategy to identify breast cancer tumor

suppressor genes. The analysis of CpG islands in gene promoter regions can now be achieved by sequencing bisulphite-modified DNA, a technique that enables the methylation status of every cytosine residue within a sequence to be determined.

Table 2 . Mutation Analysis in Breast Tumors and Cell Lines. 71 exons from 7 genes were analyzed for point mutations in the open reading frame and splice site junctions of each gene using wave. A summary of mutations and polymorphisms that were detected is presented in the table below. Mutation summary for CLCA2 is shown separately (see below). Only exons where mutations were detected are shown.

Gene	Exons	Nucleotide Change	Amino acid Change	Tumor#	Polymorphism
C1ORF29					
	Exon6	769 C to T	S256S	626, 14423, 14429, 14441	N
		779 G to A	R257H	626	N
	Exon9	-37 A to G	N	14090 636	N
GBP1					
	Exon1	-36 T to C	5'UTR	530, 581, 692, 14451	N
	Exon2	52 A to G	N 18 D	14447	N
	Exon3	232 A to G	I78V	656,660	N
	Exon6	638 G to A	S213N	14447 14435	
		793 C to A	Q265K	13253,et.al.	Y
		-10 G to A	N	530	Y
	Exon10	1522 G to C	E508Q	534	Y
IFI44					
	Exon3	-15 G to T	N	MCF7	N
	Exon4	584 T to C	I196T	MDA-MB-231	N
	Exon5	+30 C to A	N	660	N
	Exon7	1110 C to G	S370S	MDA-435	N
	Exon9	1530 G to A	In 3'UTR	620	Y
CYR61					
	Exon2	+50 C to G	N	702	N
	Exon3	-19 C to G	N	591	N
	Exon4	687 T to C	C229C	591	N
GIPC2					
	Exon1	+53 C to A	N	574	Y
	Exon3 N	-53 G to A	N	534	Y
	Exon4	617 T to C	L206P	587	Y
	Exon6	+32 C to T	N	583	Y
GBP2					
	Exon1	-69 C to T	N	689	N
	Exon7	907 A to G	S303G	530,581, 626,694	N
	Exon9	1414 G to C	A472P	620	N
	Exon11	+94 C to T	N	692	N

(+) nucleotide number after the 3' splice site; (-) nucleotide number before 5' splice site.

One way to establish whether the inactivation of a given gene is caused by promoter methylation is to treat the cells in culture with a global demethylating agent such as 5-aza-2'-dC and monitor the expression of this gene using RT-PCR.

Cell lines and 5-aza-2'-dC treatment. Breast cancer cell lines were routinely maintained in DMEM supplemented with 10% FBS at 37°C, 10% CO₂. The demethylating agent 5-aza-2'-dC (Sigma, MO) was freshly prepared in double distilled H₂O and filter sterilized. Cells (5-10x10⁵) were plated in a 100-cm² tissue culture dish in DMEM supplemented with 10% FBS at 37°C, 10% CO₂. The next day, cells were treated with 10 µM of 5-aza-2'-dC. The medium containing the demethylating agent was replaced every day for 10 days. After 10 days of treatment total RNA was prepared using TRIzol (Invitrogen, CA) according to the manufacturer's instructions. Total genomic DNA was prepared using proteinase K digestion followed by chloroform extraction and ethanol precipitation. The results are shown in Figure 4 for five genes. None of these 1p31 genes showed significant reactivation. During the course of this analysis, however, we found a high degree of methylation in the CLCA2 gene which was pursued during the remainder of the grant.

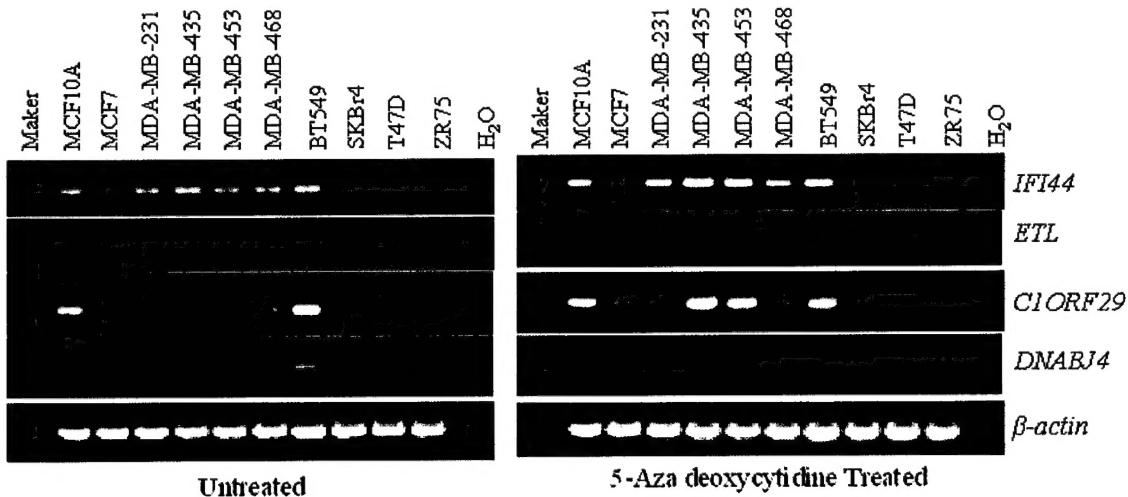


Figure 4. Effect of 5-Aza-2'-dC treatment on the restoration of gene expression. Expression of candidate genes from the 1p31 critical region 10 breast cancer cell lines before and after treatment with the demethylating agent 5-Aza-2'-dC (10 µM) for 10 days. For the four genes shown in this figure, gene expression was restored in at least one cell line after treatment of cells in culture with the demethylating agent 5-Aza-2'-dC. Gene expression was detected by RT-PCR from total RNA extracted from treated or untreated cells. Amplification of β-actin was used to normalize the cDNA concentration.

THE CLCA2 GENE.

Since none of the genes located in the smallest LOH region could be implicated in breast cancer development by mutation or methylation studies, we extended our search for genes to a slightly broader region surrounding the LOH region. During this screen, we identified the *CLCA2* gene as being down regulated in several primary breast tumors and cell lines (Figure 5). To extend these studies, we analyzed 12 invasive breast carcinomas and corresponding normal tissue that were obtained at the time of surgical resection. These tumors were detected either by mammographic screening or had presented symptomatically. None of the tumors occurred in women with a known family history of breast cancer or other cancers. All tissues were microdissected free of normal surrounding tissue, and parallel sections were used for histological characterization. In addition, DNA obtained from an additional 46 breast tumors/normal matched pairs (frozen tissue) and nine breast cancer cell lines were included.

To investigate the expression distribution of the *CLCA2* gene, we performed RT-PCR analysis of RNA from various human adult and fetal tissues. *CLCA2* is primarily expressed in the breast and lung (Figure 5), although a detectable PCR product is also seen in the placenta. We also analyzed nine breast cancer cell lines, including the immortalized MCF10A cell line which is derived from normal breast epithelium, and is widely accepted as a non tumorigenic control. 12 primary breast tumors and their matching normal tissues were also analyzed for expression of *CLCA2* using RT-PCR. Only four of nine cell lines, including MCF10A (Figure 5), and three primary tumors out of the 12 analyzed (Figure 5) showed *CLCA2* RNA expression. None of the primary tumors or cell lines which showed RNA expression had mutations in the coding sequence of the *CLCA2* gene (Li et al 2003).

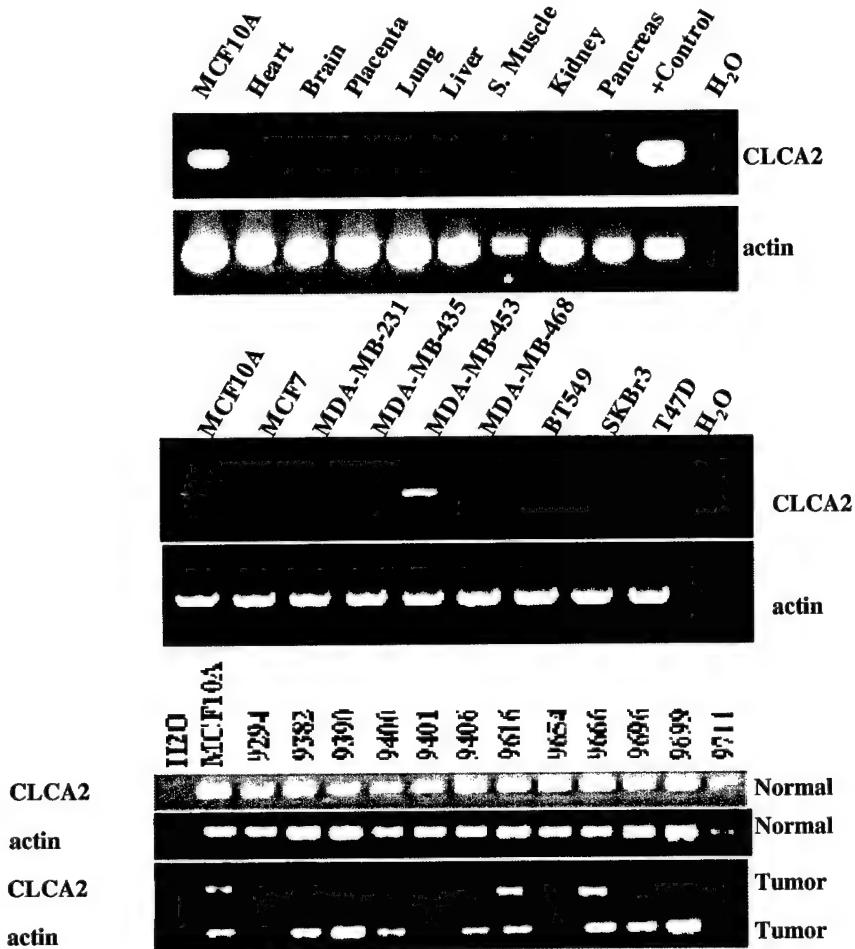


Figure 5 *CLCA2* is differentially expressed in human tissues, and is down regulated in breast cancer. Expression profile of *CLCA2* using RT-PCR analysis of total RNA from adult human tissues (top). IMAGE clone 591515 was used as the positive control for the amplification of *CLCA2* transcripts. Expression analysis of *CLCA2* in 9 breast cancer cell lines (middle). Expression analysis of *CLCA2* in 12 matched breast tumor/normal pairs (bottom). MCF10A was used as positive control for the amplification of *CLCA2* in all experiments (see text). Amplification of actin was used to normalize cDNA concentration.

We next analyzed the 14 coding exons from a total of 58 breast tumors and nine cell lines using WAVE™ DHPLC as described above. We detected three different missense substitutions in four primary breast tumors (Table 3). None of these substitutions were found in the matching normal DNA or in 200 control chromosomes. The Q306E missense mutation was detected in two breast tumors, including tumor 9711, and the D326N missense mutation was detected in tumor 9699. Both these mutations are located either close to (Q306E) or within (D326N) an evolutionary conserved functional domain i.e., the von Willebrand factor (vWF) type A domain (VWA). Furthermore, both the 9711 and 9699 tumors, where missense mutations were found, did not show any RNA expression of *CLCA2*. The Q812R missense mutation seen in tumor 534 (Table 3) does not map to any known functional domain in the *CLCA2* protein, but changes an evolutionary conserved glutamine to arginine (Li et al 2003).

The G to A substitution, located 10 nucleotides before the accepting splice site of exon 4, was detected in three primary breast tumors but not in their matching normal DNAs or in 200 normal chromosomes (Table 3). Tumor 590 has a second nucleotide substitution (T to G) only 4 nucleotides upstream of the acceptor splice site of exon 5. Two conserved changes which did not affect the amino acid at the location of the nucleotide change (A334A and R586R) were detected in three other primary breast tumors (Table 3). These changes were also detected in normal DNAs and, therefore, were considered polymorphisms rather than disease causing

mutations. Finally, a polymorphic intronic nucleotide substitution was found in four tumors as well as in several normal samples (Table 3). These results suggest protein altering mutations in CLCA2 in breast cancer although the functional consequences of these changes cannot be determined from this analysis alone. It is clear however, that mutation is not a common inactivating mechanism that might be predicted from LOH observations. We therefore undertook an analysis of the promoter region of CLCA2 to determine whether epigenetic changes were responsible for inactivation.

Table 3. Summary of mutations in *CLCA2* gene in primary breast tumors

Tumor	Exon	Nucleotide change	Predicted effect on protein
Amino acid Mutations that are likely to affect the function of CLCA2 protein			
530	6	916 C to G	Q 306 E
9711	6	916 C to G	Q 306 E
9699	7	976 G to A	D 326 N
534	14	2435 A to G	Q 812 R
Nucleotide changes with a likely effect on the splicing of the <i>CLCA2</i> transcript			
590	4	-10 G to A	None
660	4	-10 G to A	None
9294	4	-10 G to A	None
590	5	-4 T to G	None
Neutral amino acid changes with no likely effect on the function of the CLCA2 protein			
534	7	1002 C to T	A 334 A
9406	7	1002 C to T	A 334 A
9294	11	1788 C to T	R 586 R
Polymorphic changes with no likely effect on the function of the CLCA2 protein			
636	12	-43 C to T	None
9400	12	-43 C to T	None
9401	12	-43 C to T	None
9711	12	-43 C to T	None

Neither the MDA-MB-231 or MDA-MB-435 breast cancer cell lines showed expression of *CLCA2* when analyzed by RT-PCR (Figure 5). However, treatment of these two cell lines with the demethylating agent 5-aza-2'-dC was able to restore *CLCA2* expression to levels that could be detected by RT-PCR (Figure 6). This observation prompted us to analyze the methylation status of the *CLCA2* promoter region in these cell lines. The *CLCA2* promoter region was determined by the presence of a CpG island using the PromoterInspector software. After bisulfate modification of cell line DNA, and PCR amplification of the *CLCA2* promoter region, at least 10 alleles for each cell line were sequenced.

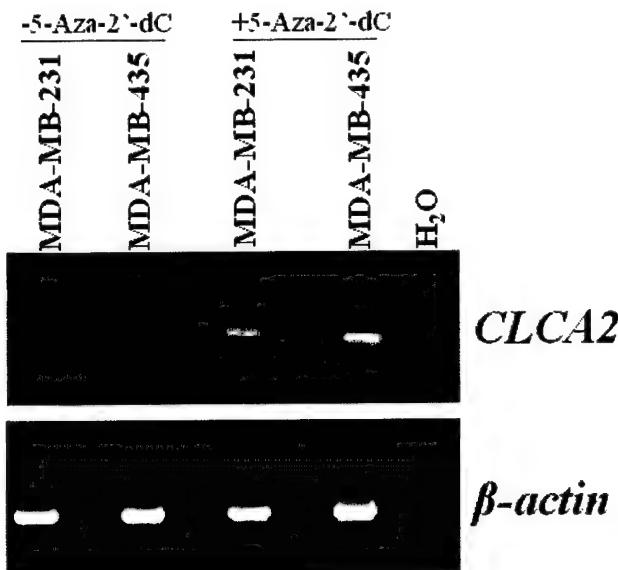


Figure 6. Promoter region methylation silences CLCA2 expression. Expression of CLCA2 in two breast cancer cell lines before and after treatment with the demethylating agent 5-Aza-2'-dC (10 uM) for 10 days. Expression of CLCA2 was detected by RT-PCR from total RNA extracted from treated or untreated cells. Amplification of actin was used to normalize the cDNA concentration.

We also sequenced bisulfate-treated DNA from 12 primary breast tumors and found that they also exhibited tumor-specific hypermethylation (Li et al 2003). The *CLCA2* promoter hypermethylation correlated with the presence of expression, and all cell lines with promoter methylation showed reduced or absent *CLCA2* RNA expression. Cell lines and primary tumors with apparently normal *CLCA2* expression levels did not exhibit hypermethylation of the promoter

(Li et al 2003). Furthermore, methylation of the *CLCA2* promoter region was considerably reduced in cell lines treated with the demethylating agent 5-aza-2'-dC (Figure 6), while the expression of *CLCA2* RNA was restored to normal levels. *CLCA2* methylation was not detected in two cell lines derived from normal lymphocytes, one of which is shown as the normal control in Figure 5.

We have thus demonstrated that hypermethylation of the *CLCA2* promoter is the major cause of *CLCA2* silencing in breast cancer. We have demonstrated that the promoter region is frequently methylated in breast tumors and cell lines using bisulfate DNA sequencing. We also demonstrated that missense mutations in the coding sequence of *CLCA2* can be detected, although to a much lesser extent, in breast tumors. Our results supports the role of *CLCA2* as a tumor suppressor gene from the 1p31 region that is apparently deleted in more than 50% of sporadic breast tumors.

KEY RESEARCH ACCOMPLISHMENTS:

- Cloning and characterization of the TTC4 gene from 1p31.
- Exon by exon mutation analysis for the TTC4 gene in 28 breast tumors and cell lines.
- Construction of a BAC contig from the extended region of LOH in 1p31.
- Analysis of approximately 1700 kb of sequence using GenScan to predict genes in the critical region.
- Identification of ESTs and Unigene clusters in the 1p31 region
- Exclusion of the progesterone receptor gene in the development of breast cancer
- Expression studies of known genes in the critical 1p31 region identified those whose expression is downregulated in BRCA.
- Mutation analysis of down regulated genes did not identify mutations
- Demonstration that the CLCA2 gene in 1p31 is inactivated in breast cancer cell lines and tumors
- Identification of infrequent mutations in CLCA2 in breast cancer
- Demonstration that promoter methylation is the main cause of inactivation of CLCA2 in breast cancer
- Identification of CLCA2 as the most likely gene in 1p31 to be involved in the development of breast cancer.

REPORTABLE OUTCOMES/

BIBLIOGRAPHY OF ALL PUBLICATIONS AND RESEARCH ABSTRACTS

Abstracts

Li X, Sossey-Alaoui K, Head K, Asch H, Cowell JK. CLCA1, a CA2+-activated C1-channel is mutated and down regulated in breast cancer. Proceedings of the American Association for Cancer Research, 44: Abstr. #6076, pg. 1394, 2003

Sossey-Alaoui K, Kitamura E, Su G, Jacobs B, Casey G, Borden E, Cowell JK. Mutation analysis of TTC4, a novel tetratricopeptide repeat-containing gene, in malignant melanoma of the skin. Proceedings of the American Association of Cancer Research, 42: Abstr. #2861, pg. 531, 2001.

Journals

Li X, Sossey-Alaoui K, Cowell JK. The CLCA2 tumour suppressor gene in 1 p31 is epigenetically regulated in breast cancer. *Oncogene*, In Press.

Su G, Roberts T, Cowell JK. (1999) TTC4, a novel human gene containing the tetratricopeptide repeat and which maps to the region of chromosome 1p31 which is frequently deleted in sporadic breast cancer. *Genomics* 55; 157-163.

Sossey-Alaoui K, Kitamura E and Cowell JK. (2001) Fine mapping of the PTGFR gene to 1p31 region and mutation analysis in human breast cancer. *Int. J. Molec. Med.* 7; 543-54.

CONCLUSIONS

Loss of heterozygosity in 1p31 is a major event in the development of breast cancer. Despite extensive screening of genes for inactivation and mutation none of the genes in the minimum region of overlap could be identified as the critical gene. It is possible that there are as yet unidentified genes in this region which cannot be predicted using current bioinformatics tools. Despite being good candidates based on their function the TTC4 and PTGFR genes do not seem to be the tumor suppressor gene in this region. By extending our region in 1p31 we identified the CLCA2 gene which is normally expressed in breast tissues and inactivated in a large number of breast tumors. Mutation screening identified protein altering mutations that did not appear to be polymorphisms. From the sequence change alone it is not possible to determine whether these are responsible for inactivation of gene function and this will involve more extensive functional studies where the effect on breast cancer cells is assessed after transfer of the normal and mutant genes. These studies were beyond the scope of this current study. Inactivation of CLCA2, however, was demonstrated to be due to methylation of the promoter in a large percentage of tumors which raises the possibility that reactivation of this gene using demethylation agents may provide a novel means of treating breast cancer. Again this analysis was beyond the scope of the present study. The CLCA2 gene encodes a calcium channel but also appears to have tumor suppressor function and a more detailed analysis of the function of this gene is warranted to understand more about its role in breast cancer development.

LIST OF PERSONNEL

John K. Cowell, Ph.D.

Xiurong Li, Ph.D.

APPENDICES



ORIGINAL PAPER

CLCA2 tumour suppressor gene in 1p31 is epigenetically regulated in breast cancer

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The calcium-activated chloride channel gene family is clustered in the 1p31 region, which is frequently deleted in sporadic breast cancer. Recent studies have indicated the association of the second member of this gene family (*CLCA2*) with the development of breast cancer and metastasis. We have now shown the absence of expression of *CLCA2* in several breast cancer tumours and cell lines, which confirms the results from other reports. When overexpressed in *CLCA2*-negative cell lines, their tumorigenicity and metastasis capability were significantly reduced, suggesting a tumour suppressor role for *CLCA2* in breast cancer. The mechanisms behind the silencing of *CLCA2* in breast cancer, however, have not been elucidated to date. Although we were able to identify *CLCA2* mutations in breast cancers, somatic mutations are not the major cause of *CLCA2* gene silencing. On the other hand, treatment of breast cancer *CLCA2*-negative cells lines with demethylating agents was able to restore *CLCA2* expression, suggesting an epigenetic inactivation of this gene. Bisulphite-sequencing of the promoter-associated CpG island of the *CLCA2* gene in breast tumours demonstrated that the absence of expression in these tumours was caused by hypermethylation of the promoter CpG island. In contrast, in breast cancer cells lines, tumours, and control cell lines that express *CLCA2*, a much lower level, and often absence, of methylation of the promoter were demonstrated. These findings demonstrate that *CLCA2* is frequently inactivated in breast cancer by promoter region hypermethylation, which makes it an excellent candidate for the 1p31 breast cancer tumour suppressor gene.

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Keywords: *CLCA2*; tumour suppressor gene; 1p31; breast cancer; epigenetics

Introduction

Overall, breast cancer accounts for 32% of all new cases of cancer in women. The majority of hereditary cases of

breast cancers can be accounted for by germline mutations in *BRCA1* and *BRCA2* genes (Antoniou *et al.*, 2003). Only 5–10% of cases, however, have a family history of tumours and, in the 90% of breast cancer cases which occur sporadically, both the *BRCA1* and *BRCA2* genes have been shown not to be involved. To date, several other genes, with tumour suppression activity, have been identified to be involved in the development and progression of breast cancer.

Positional cloning has been the most successful strategy for isolating tumour suppressor genes to date (Collins, 1992). This approach, however, requires that the chromosomal location of the gene is known. Possibly, the most powerful indicator of the location of these genes in sporadic tumours has come from loss of heterozygosity (LOH) studies (Cavenee *et al.*, 1983). Once a small region of frequent LOH has been identified, it is then possible to use positional cloning strategies to isolate candidate genes from the region. Although there is evidence that genes in chromosome regions 11p15 (Lichy *et al.*, 1998; Roy *et al.*, 2003) and 17p13 (Hoff *et al.*, 2001) may be important in breast cancer tumorigenesis, chromosome 1 is the most frequently involved in rearrangements in sporadic tumours (Hoggard *et al.*, 1995; Nagai *et al.*, 1995; Tsukamoto *et al.*, 1998). These aberrations frequently result in loss of material from the short arm of chromosome 1 (1p). Molecular studies have indicated that at least 50% of tumours show LOH for genetic markers on 1p (Antoniou *et al.*, 2003), and recently it has been shown, using microsatellite markers, that LOH of the 1p31 region occurs in 60% of breast tumours (Hoggard *et al.*, 1995). The common region of overlap is reportedly between the *D1S207* and *D1S2876* loci, with an estimated genomic size of 3.5 Mbp, and contains several known and predicted genes (White *et al.*, 1998; Su *et al.*, 1999; Sossey-Alaoui *et al.*, 2001).

In higher eukaryotes, DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide (Holliday and Grigg, 1993). This modification has important regulatory effects on gene expression, especially when CpG-rich regions, located in the promoter regions of many genes are involved (Bird, 2002). Aberrant methylation of the normally unmethylated CpG islands has been documented as a relatively frequent event in immortalized and transformed cells (Antequera *et al.*, 1990), and has been associated with transcriptional inactivation of defined tumour suppres-

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Q1

Q2

sor genes in human cancers (Esteller, 2002). In this case, instead of coding region mutations, promoter region hypermethylation provides an alternative means of eliminating tumour suppressor gene function. Analysing the methylation status of CpG islands of genes from the 1p31 region, therefore, is an important strategy to identify breast cancer tumour suppressor genes. The analysis of CpG islands in gene promoter regions can now be achieved by sequencing bisulphite-modified DNA, a technique that enables the methylation status of every cytosine residue within a sequence to be determined (Frommer *et al.*, 1992; Clark *et al.*, 1994; Grigg and Clark, 1994).

During our screening for breast cancer candidate genes from chromosome region 1p31, we identified the *CLCA2* gene as being downregulated in several primary breast tumours and cell lines, confirming the findings reported by Gruber and Pauli (1999). In the present report, we show that it is the hypermethylation of the *CLCA2* promoter that is the major cause of *CLCA2* silencing in breast cancer. We demonstrate that the promoter region is frequently methylated in breast tumours and cell lines using bisulphite DNA sequencing. We also demonstrate that missense mutations in the coding sequence of *CLCA2* can also be detected, although to a much lesser extent, in breast tumours. Our results support the role of *CLCA2* as a tumour suppressor gene from the 1p31 region that is apparently deleted in more than 50% of sporadic breast tumours.

Results

CLCA2 is downregulated in breast cancer

To investigate the expression distribution of the *CLCA2* gene, we performed RT-PCR analysis of RNA from various human adult and fetal tissues. *CLCA2* is primarily expressed in the breast and lung (Figure 1a). This result confirms the previously reported data (Agnel *et al.*, 1999; Gruber *et al.*, 1999). *CLCA2* expression is also detected in the placenta (Figure 1a). We also analysed nine breast cancer cell lines, including the immortalized MCF10A cell line, that is derived from normal breast epithelium, and is widely accepted as nontumorigenic breast control (Soule *et al.*, 1990). A total of 12 primary breast tumours and their matching normal tissues were also analysed for expression of *CLCA2* using RT-PCR. Only four out of nine cell lines, including MCF10A (Figure 1b), and three primary tumours out of the 12 analysed (Figure 1c) showed *CLCA2* RNA expression. Similar results were reported by Gruber and Pauli (1999). None of the primary tumours or cell lines that showed RNA expression had mutations in the coding sequence of the *CLCA2* gene (see below).

CLCA2 gene is not commonly mutated in breast cancer

We analysed the 14 coding exons from a total of 58 breast tumours and nine cell lines using WAVE™

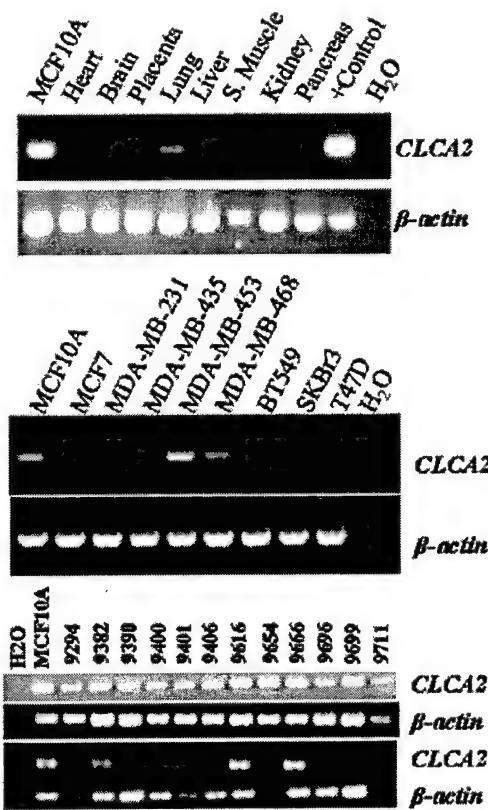


Figure 1 *CLCA2* is differentially expressed in human tissues, and is downregulated in breast cancer. (a) Expression profile of *CLCA2* using RT-PCR analysis of total RNA from adult human tissues. IMAGE clone 591515 was used as the positive control for the amplification of *CLCA2* transcripts. (b) Expression analysis of *CLCA2* in nine breast cancer cell lines. (c) Expression analysis of *CLCA2* in 12 breast tumour. MCF10A was used as positive control for the amplification of *CLCA2* in all experiments (see text). Amplification of *β-actin* was used to normalize cDNA concentration

DHPLC as described previously (Sossey-Alaoui *et al.*, 2002b). We detected three different missense substitutions in four primary breast tumours (Table 1). None of these substitutions were found in the matching normal DNA or in 200 control chromosomes. The Q306E missense mutation was detected in two breast tumours, including tumour 9711, and the D326N missense mutation was detected in tumour 9699 (Table 1, Figure 2b). Both these mutations are located either close to (Q306E) or within (D326N) an evolutionarily conserved functional domain, that is, the von Willebrand factor (vWF) type A domain (VWA) (Figure 2c). Furthermore, both the 9711 and 9699 tumours, where missense mutations were found (Table 1), did not show any RNA expression of *CLCA2* (Figure 1c). The Q812R missense mutation seen in tumour 534 (Table 1, Figure 2b) does not map to any known functional domain in the *CLCA2* protein, but changes an evolutionarily conserved glutamine to arginine (Figure 2c).

Table 1 Summary of mutations in *CLCA2* gene in primary breast tumours

Tumour	Exon	Nucleotide change	Predicted effect on protein
<i>Amino-acid mutations that are likely to affect the function of CLCA2 protein</i>			
530	6	916 C to G	Q306E
9711	6	916 C to G	Q306E
9699	7	976 G to A	D326N
534	14	2435 A to G	Q812R
<i>Nucleotide changes with a likely effect on the splicing of the CLCA2 transcript</i>			
590	4	-10 G to A	None
660	4	-10 G to A	None
9294	4	-10 G to A	None
590	5	-4 T to G	None
<i>Neutral amino-acid changes with no likely effect on the function of the CLCA2 protein</i>			
534	7	1002 C to T	A334A
9406	7	1002 C to T	A334A
9294	11	1788 C to T	R586R
<i>Polymorphic changes with no likely effect on the function of the CLCA2 protein</i>			
636	12	-43 C to T	None
9400	12	-43 C to T	None
9401	12	-43 C to T	None
9711	12	-43 C to T	None

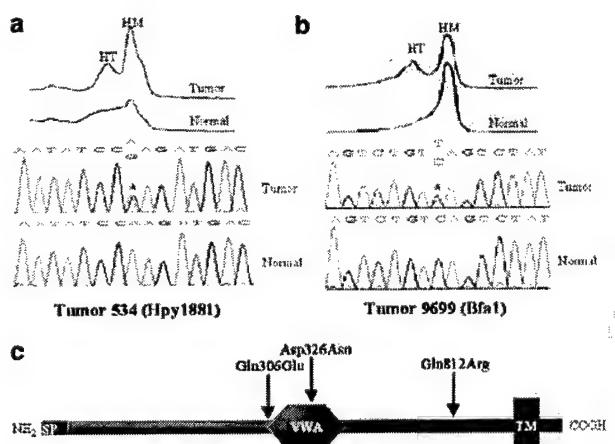


Figure 2 WAVE™-DHPLC mutation analysis of *CLCA2* in breast tumour 534 (a) and tumour 9699 (b). In the absence of mutations, the two alleles of *CLCA2* form a homoduplex (HM), which elutes from the column at the same point. Any mismatch due to mutations leads to the formation of a heteroduplex (HT), which elutes earlier. Only homoduplexes are detected in the normal DNAs, but the corresponding tumour DNAs show heteroduplexes. When the DNAs showing the heteroduplexes were sequenced, the presence of a heterozygous base change was seen (double peak at *). Tumour 534 has an A 2435 G substitution in exon 14 (Gln812Arg), and tumour 9699 has a G 976 A substitution in exon 7 (Asp326Asn). The sequence of the opposite strand of exon 7 is shown. The restriction enzymes that were used to verify the authenticity of the mutations are given in parenthesis. (c) Schematic diagram of the *CLCA2* protein and its domains as predicted using the SMART software (<http://smart.embl-heidelberg.de>). SP, signal peptide; VWA, von Willebrand factor (vWF) type A domain; TM, transmembrane domain. The locations of the missense mutations are indicated by arrows.

The G to A substitution, located 10 nucleotides before the accepting splice site of exon 4, was detected in three primary breast tumours, but not in their matching normal DNAs or in 200 normal chromosomes (Table 1).

Tumour 590 has a second nucleotide substitution (T to G) only four nucleotides upstream of the acceptor splice site of exon 5. Two conserved changes, which did not affect the amino acid at the location of the nucleotide change (A334A and R586R), were detected in three other primary breast tumours (Table 1). These changes were also detected in normal DNAs and, therefore, were considered polymorphisms rather than disease causing mutations. Finally, a polymorphic intronic nucleotide substitution was found in four tumours as well as in several normal samples (Table 1).

Promoter hypermethylation is the major cause of the *CLCA2* gene silencing in breast cancer tumours and cell lines

Neither the MDA-MB-231 nor MDA-MB-435 breast cancer cell lines showed expression of *CLCA2* when analysed by RT-PCR (Figure 1b). However, treatment of these two cell lines with the demethylating agent 5-aza-2'-dC was able to restore *CLCA2* expression to at least 50% of the expression level of β -actin (Figure 3a). Cell-line treatment with 5-aza-2'-dC was also able to restore the expression of *CLCA2* in MCF7, SKBr3 and T47D cell lines (Figure 3b), although to levels much lower compared to the ones observed in MDA-MB-231 and MDA-MB-435. A noticeable increase in the expression levels of *CLCA2* was also detected in the MDA-MB-468 and BT549 cell lines (Figure 3b). This observation prompted us to analyse the methylation status of the *CLCA2* promoter region in these cell lines. The *CLCA2* promoter region was determined by the presence of a CpG island upstream of the first coding exon of *CLCA2*, using the PromoterInspector software (Figure 4a, b). After bisulphite modification of cell-line genomic DNA, and PCR amplification of the *CLCA2* promoter region, at least 10 alleles for each cell line were sequenced (Figure 5a).

Epigenetic silencing of *CLCA2* in breast cancer
X Li et al

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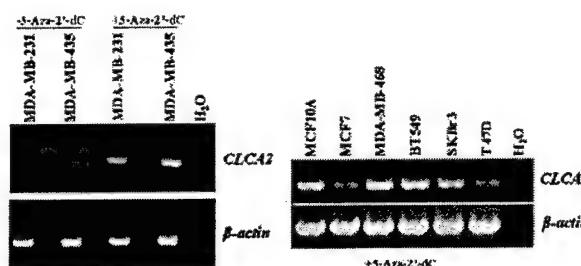


Figure 3 5-Aza-2'-dC treatment restores *CLCA2* expression. Expression of *CLCA2* in MDA-MB-231 and MDA-MB-435 breast cancer cell lines before and after treatment with the demethylating agent 5-aza-2'-dC (10 µM) for 10 days (a). (b) Expression of *CLCA2* in six other cell lines after treatment with 5-aza-2'-dC. *CLCA2* expression before the treatment is shown in Figure 1b. Expression of *CLCA2* was detected by RT-PCR from total RNA extracted from treated or untreated cells. Amplification of β-actin was used to normalize the cDNA concentration

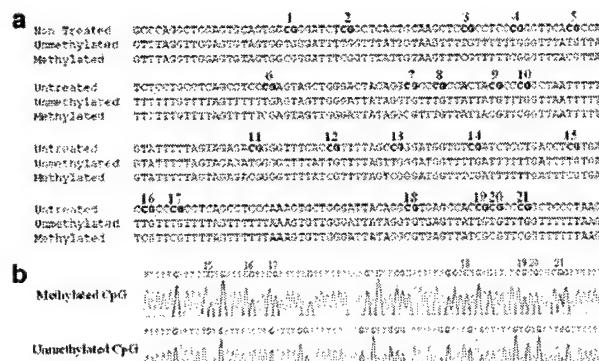


Figure 4 Example of direct DNA sequencing chromatogram of the methylated and unmethylated alleles. (a) Nucleotide sequence of the fragment used for methylation analysis depicting the original sequence (not treated with bisulphite), and the sequence after bisulphite treatment (unmethylated and methylated). The numbers, 1–21, indicate the position of each CpG dinucleotide. (b) Chromatograms of methylated and unmethylated alleles after bisulphite treatment showing that the CpG cytosines remain unaltered if methylated, whereas unmethylated CpG cytosines and all other cytosines are deaminated and converted to thymidines

We also sequenced bisulphite-treated DNA from the 12 primary breast tumours, described above, and found that they also exhibited tumour-specific hypermethylation (Figure 5b). The *CLCA2* promoter hypermethylation clearly correlated with the presence of expression of *CLCA2* in cell lines and tumours. Furthermore, all cell lines with promoter methylation showed reduced or absent *CLCA2* RNA expression, whereas cell lines and primary tumours with apparently normal *CLCA2* expression levels did not exhibit hypermethylation of the promoter. At the same time, methylation of the *CLCA2* promoter region was considerably reduced in cell lines treated with the demethylating agent 5-aza-2'-dC (Figure 5a), while the expression of *CLCA2* RNA was restored. *CLCA2*-promoter methylation was not detected in two samples, derived from normal breast tissue, as well as in MCF10A cell line.

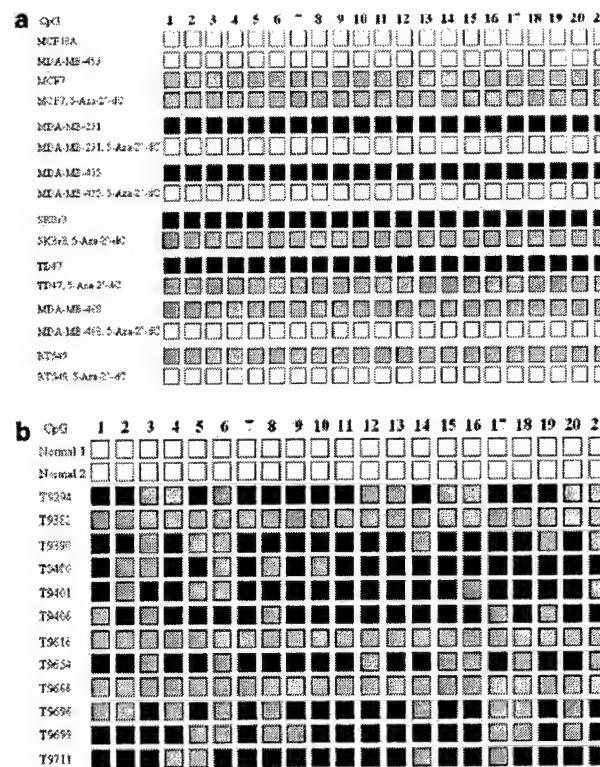


Figure 5 Methylation analysis of the CpG island of the *CLCA2* gene in breast cancer. Genomic DNA was isolated from the indicated tumours or cell lines and analysed for methylation by direct bisulphite sequencing. Bisulphite-PCR products from breast cancer cell lines (a), and from normal breast and breast tumours (b) were subcloned into the pCR21 vector and sequenced. The ratio of all methylated sites from 10 clones was determined and recorded as a percentage. The methylation status of CpG residues 1–21 is indicated by shading, black (methylated), white (unmethylated) and grey (partially methylated)

Discussion

Aberrant methylation of normally unmethylated promoter-associated CpG islands has commonly been studied as the epigenetic mechanism associated with the transcriptional silencing of known and putative tumour suppressor genes (Baylin and Herman, 2000; Garinis et al., 2002). Selected examples include the BRCA1, ER, PR, p16, SLIT2, FHIT and LOT1 genes, which have now been identified to be epigenetically silenced in breast cancer (Cattaneo et al., 1999; Zochbauer-Muller et al., 2001; Dallop et al., 2002; Esteller, 2002; Abdollahi et al., 2003). Hypermethylation of gene promoter regions is now widely accepted as being responsible for tumour suppressor gene silencing in a variety of human cancers (Garinis et al., 2002).

The *CLCA2* gene family is a novel family of calcium-activated chloride channels (Pauli et al., 2002). Several members of this family have been cloned from different mammalian species with distinct, highly specific expression patterns (Gruber et al., 1998, 1999). All four members of the human *CLCA* gene family cluster on the

short arm of chromosome 1 at 1p31, a region that is frequently deleted in breast cancer (Hoggard *et al.*, 1995; Nagai *et al.*, 1995; Tsukamoto *et al.*, 1998; Su *et al.*, 1999; Sossey-Alaoui *et al.*, 2001). However, only *CLCA2* gene expression was shown to be downregulated in breast cancer (Gruber and Pauli, 1999, this study). Loss of *CLCA2* expression in human breast cancer appears to be closely associated with tumorigenicity, because neither breast tumours nor tumorigenic breast cancer cell lines were found to express *CLCA2* (Figure 1). On the other hand, normal breast epithelial cells and the spontaneously immortalized, non-tumorigenic normal epithelial breast cells (MCF10A) all express *CLCA2* (Figure 1). Furthermore, when *CLCA2* expression was re-established in breast cancer cell lines, their tumorigenicity was attenuated (at least for MDA-MB-231 and MBA-MD-435 cell lines), as judged by the results of anchorage-independent growth, migration, and *in vitro* invasion suppression assays (Elble and Pauli, 2001). All these experiments strongly suggest that *CLCA2* is involved in the development of breast cancer. The mechanism behind the suppression of *CLCA2* activity, however, has not been elucidated to date. The purpose of this study was to determine the molecular mechanisms that are responsible for the silencing of the *CLCA2* gene in breast cancer. We have shown the presence of missense mutations in the coding region of the *CLCA2* gene in a small group of tumours (Table 1, Figure 2). These mutations are located in evolutionarily conserved functional domains and so are likely to affect the function of *CLCA2*. We have also identified two nucleotide substitutions that are located within 10 nucleotides or less of the exon splice junctions. These mutations could possibly alter the splicing of the *CLCA2* transcript and, therefore, suppress its function by generating an aberrant, unstable splice form of the *CLCA2* transcript. Unfortunately, in the absence of tissue material from the tumours where these mutations were found, it remains difficult to verify this hypothesis. Most of the nucleotide mutations that we found in breast tumours were detected in heterozygous form. This could be explained by the presence of contaminating normal tissue with the tumour during the time the samples were collected. Overall, nucleotide substitutions in the *CLCA2* gene are not the major reason for alteration of *CLCA2* function in breast tumours, since they are not frequent events.

We have shown that treatment of breast cancer cell lines, which do not express *CLCA2* (see Figure 1b), with the demethylating agent 5-aza-2'-dC was able to restore the expression of *CLCA2*, even though to different degrees, in all five cell lines (Figure 3a, b). 5-Aza-2'-dC treatment was also able to noticeably increase the expression levels of *CLCA2* in cell lines that normally express this gene, namely MDA-MB-468 and BT549 (Figure 3b). This observation strongly supports the idea that the silencing of the *CLCA2* gene is due to DNA methylation. We have identified a CpG island in the upstream sequence of exon 1 and into the promoter of the *CLCA2* gene, as determined by sequence analysis using the PromotorInspector software, which suggest

that methylation in CpG dinucleotides may play a role in silencing gene expression. We confirmed this hypothesis by sequencing bisulphite-treated DNA from breast cancer cell lines and breast tumours (Figure 5a, b). The degree of *CLCA2*-CpG methylation in breast tumours and cell lines clearly correlates with the levels of expression of *CLCA2* in these samples. All the breast tumours and cell lines, where no, or very low expression of *CLCA2*, was detected, showed hypermethylation in the promoter CpG island. One exception to this rule is observed for MCF7, where there is no clear correlation between the expression of *CLCA2* and the methylation status of the promoter CpG island in this cell line. One explanation for this lack of correlation in MCF7 could come from the fact that the alteration of the function of a single gene, such as *CLCA2*, cannot account for all the breast cancer cases, including MCF7. On the other hand, all the breast tumours and cell lines, which express *CLCA2*, had low or no methylation of the promoter CpG island.

In conclusion, aberrant promoter methylation and associated transcriptional silencing are now recognized as a major mechanism of tumour suppressor gene inactivation. *CLCA2* resembles other tumour suppressor genes, such as SLIT2 (Dallo *et al.*, 2002), LOT1 (Abdollahi *et al.*, 2003) and RASSF1A (Dammann *et al.*, 2003), in that epigenetic inactivation appears to be much more frequent than mutational mechanisms. *CLCA2* is a calcium-activated chloride channel, the exact function of which is still unclear. It has been demonstrated that the activity of chloride channels, like *CLCA2* varies during the cell cycle. Chloride current peaks in G1 and is lowest in S phase (Bubien *et al.*, 1990; Ullrich and Sontheimer, 1997). Moreover, the blockade of chloride channels was found to enhance cell proliferation (Deane and Mannie, 1992). Suppression of the chloride channel activity of *CLCA2* in the breast epithelium could, therefore, be responsible for the enhancement of abnormal cell proliferation, which ultimately leads to the development of a malignant phenotype. A more detailed biochemical, molecular and cellular analysis of the expression of *CLCA2* and its impact on the cell cycle progression will be needed to provide a clearer understanding to its role in breast cancer.

Materials and methods

Tissue samples

We analysed a total of 12 invasive breast carcinomas and corresponding normal tissue that were obtained at the time of surgical resection. These tumours were detected either by mammographic screening or had presented symptomatically. None of the tumours occurred in women with a known family history of breast cancer or other cancers. All tissues were microdissected free of normal surrounding tissue, although a contamination with surrounding normal breast tissue could not be ruled out. In addition, DNA obtained from an additional 46 breast tumours (frozen tissue) and nine breast cancer cell lines was analysed for *CLCA2* mutations and promoter CpG island methylation. We also used as controls, DNA extracted from lymphocytes of normal individuals.

Cell lines and 5-aza-2'-dC treatment

Breast cancer cell lines were routinely maintained in DMEM supplemented with 10% FBS at 37°C, 10% CO₂. The demethylating agent 5-aza-2'-dC (Sigma, MO, USA) was freshly prepared in double-distilled H₂O and filter sterilized. Cells (5–10 × 10⁵) were plated in a 100-cm² tissue culture dish in DMEM supplemented with 10% FBS at 37°C, 10% CO₂. The next day, cells were treated with 10 µM of 5-aza-2'-dC. The medium containing the demethylating agent was replaced every day for 10 days. After 10 days of treatment, total RNA was prepared using TRIzol (Invitrogen, CA, USA), according to the manufacturer's instructions. The total genomic DNA was prepared using proteinase K digestion as previously described (Sossey-Alaoui *et al.*, 2002a).

Expression analysis of CLCA2

CLCA2 expression was detected by RT-PCR using the primers 5'-TTCTTGGCAATTGCTTACCC-3' and 5'-CATAGTGTGTTGCCAACAG-3' with an expected PCR product size of 298 bp. Expression of β-actin was used as a control. The β-actin primers were 5'-TGCTTGTAACCCATTCTCT-TAA-3' and 5'-GGATCTTCATGAGGTAGTCAGTC-3' with an expected PCR product size of 629 bp. We used total RNA extracted from primary breast tumours as well as from untreated and 5-aza-2'-dC treated breast cancer cell lines to monitor the expression of *CLCA2*. Panels of normalized, first-strand cDNA preparations from RNA from various human and mouse tissues were purchased from BD Biosciences (CA) and used for RT-PCR to determine the tissue distribution of *CLCA2* expression. PCR was carried out in an MJ Research Inc., Thermocycler (Waltham, MA, USA) using the following conditions: 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. A final cycle of 72°C for 5 min was applied to ensure complete elongation of the PCR products. The PCR samples were then cooled to 4°C before being resolved by agarose gel electrophoresis.

Mutation analysis of primary tumours and cell lines

Intron-exon boundaries were determined by aligning the cDNA sequence for *CLCA2* (GenBank Accession Number NM_006536) against BAC clone RP11-444C12 (GenBank Accession Number AC119749), which contains the entire sequence of the *CLCA2* gene. The WAVE instrument (Transgenomic, NE, USA) was utilized to perform denaturing

high-performance liquid chromatography (HPLC) and search for mutations in *CLCA2* as described elsewhere (Sossey-Alaoui *et al.*, 2002b). Oligonucleotide primers specific to each exon of *CLCA2* (Table 2) were used for the mutational screening of primary breast tumours and cell lines. Owing to the large size of exons 6 and 14, we used two overlapping amplicons to analyse each one of these two exons (Table 2). Mutations were verified by restriction analysis in the tumour and normal DNAs, when the nucleotide substitution results in the creation or abolition of an endonuclease restriction site. In cases where the mutation does not alter the restriction profile of the tumour, an oligonucleotide primer was designed where the nucleotide adjacent to the mutation is modified in a way that, together with the mutation, will create or abolish a restriction site in the tumour but not in the normal control.

Bisulphite modification and methylation analysis

Bisulphite DNA sequencing was performed as described in Frommer *et al.* (1992), with minor adjustments to optimize the conditions to our material. Briefly, 1–2 µg of genomic DNA was denatured in 0.3 M NaOH for 30 min at 42°C, and then the unmethylated cytosine residues were sulphurated by incubation in 3.12 M sodium bisulphite (pH 5.0; Sigma)/5 mM hydroquinone (Sigma, MO, USA) at 55°C for 16 h. The sulphurated DNA was recovered using the QIAquick Gel Extraction system (Qiagen, MA, USA), according to the manufacturer's recommendations. The conversion reaction was completed by desulphurization in 0.3 M NaOH for 5 min at room temperature. The DNA was ethanol precipitated and resuspended in double-distilled water.

The *CLCA2* putative promoter region was predicted from the genomic sequence of BAC clone RP11-444C12 upstream of the first exon of *CLCA2* gene, using the PromoterInspector prediction software (<http://www.genomatix.ed>). The region (279 bp) that covers the CpG island was amplified from breast tumours and cell lines not expressing or expressing *CLCA2* using primers 5'-GGGATTATTATTGTTTATTITTA-GAT-3' and 5'-ATCTACCCACTATAATACCCCTAC-3', which were designed with the assistance of the MethPrimer software (<http://itsa.ucsf.edu/~urolab/methprimer/index1.html>). In total, 20–50 ng of bisulphite-treated DNA was used as template in each PCR reaction using the following conditions: 95°C for 5 min, followed by 40 cycles of 15 s of denaturation at 95°C, 20 s at 55°C and 25 s of extension at

Table 2 Primers for PCR amplification of *CLCA2* exons

Exon	Forward primer	Reverse primer	Product size (bp)
Exon 1	5'-gatccaccccagactttca-3'	5'-ggctgtatcaaattggatgc-3'	387
Exon 2	5'-ccaaaagttagggcttc-3'	5'-ttcccccttggatcatgtt-3'	321
Exon 3	5'-taccaacaacacaggctgaga-3'	5'-ctttcagcaccaacacaga-3'	373
Exon 4	5'-tagcaaggccatgtcgag-3'	5'-cacttaaatgttgcatttgc-3'	229
Exon 5	5'-tcccaagttcttcatttcattt-3'	5'-ggaaatctgcacaaaggatgtgc-3'	226
Exon 6a	5'-gttgctttggagaaatgtgc-3'	5'-cgagaatgtggggaggatgc-3'	217
Exon 6b	5'-cacacccaagaaggacacaaa-3'	5'-tgacatgtaccccaaaact-3'	297
Exon 7	5'-ccaaatgttgcattttttt-3'	5'-tccaaaggctttaggatgtct-3'	298
Exon 8	5'-tggaaatttggatgagatgt-3'	5'-aagggacatgtccaaaaga-3'	307
Exon 9	5'-tgcacaaaggaaatgttgc-3'	5'-tgaccatgtatgttgcgg-3'	202
Exon 10	5'-aaaaagaaaaatggatgattatg-3'	5'-agctgttgcgttggatccaa-3'	287
Exon 11	5'-gcaacatcaacaaaacaaagg-3'	5'-ccatcatcaaggatgtcagc-3'	376
Exon 12	5'-gaaaaattatggacccatgt-3'	5'-gccaccccccgtacacacata-3'	427
Exon 13	5'-tgtaaatgttgcattttgc-3'	5'-tcagttctactagccctgtc-3'	354
Exon 14a	5'-ttttgttgcgttcatttagtaat-3'	5'-cctatccatgtctgtatgc-3'	314
Exon 14b	5'-cagccaaatggagaaacaca-3'	5'-cgaaggccatgggttttat-3'	332

72°C. The PCR reaction was terminated with an additional 7 min of extension and cooled to 4°C. The PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and the 279-bp bands were excised and gel was purified using the QIAquick Gel Extraction system (Qiagen, MA, USA). The purified PCR products were cloned into the pCR2.1 vector (Invitrogen, CA, USA), and at least 10 clones were sequenced in each experiment. The methylation status at each CpG site of the CLCA2 gene promoter was analysed using the MethTools software (<http://genome.imb-jena.de/methtools/>).

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TTC4, a Novel Human Gene Containing the Tetratricopeptide Repeat and Mapping to the Region of Chromosome 1p31 That Is Frequently Deleted in Sporadic Breast Cancer

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The 1p31 region shows loss of heterozygosity in up to 50% of human breast cancers, indicating the presence of a tumor suppressor gene in this location. We have mapped six novel ESTs to a 15-Mb contig of yeast artificial chromosomes spanning the critical region of 1p31. One of these ESTs was localized within the contig to the region most commonly undergoing loss of heterozygosity in breast cancer. The corresponding gene sequence for this EST was established by cDNA cloning and RACE procedures. This gene is 2 kb long and contains a tetratricopeptide repeat motif and a coiled-coil domain. This family of genes has been implicated in a wide variety of functions, including tumorigenesis. This is the fourth member of the human gene family, and so we have named this gene TTC4. Northern blot analysis demonstrates a ubiquitous pattern of gene expression that includes breast tissue. A preliminary screen of human breast cancer cell lines shows that TTC4 is expressed in all cases, but SSCP analysis of the coding region of this gene following RT-PCR failed to reveal any mutations. Clearly, because of its map location, a more extensive analysis is warranted to establish whether subtle mutations are present in breast cancers. © 1999 Academic Press

in the regulation of cell division and RNA synthesis (Hirano *et al.*, 1990; Sikorski *et al.*, 1990), more recently it has been shown that members of this family are clearly related to physiological functions such as cellular stress responses (Nicolet and Craig, 1989; Honore *et al.*, 1992), the interferon response (Lee *et al.*, 1994), transport of proteins across both the mitochondrial (Steger *et al.*, 1990) and peroxisomal (Brocard *et al.*, 1994; Fransen *et al.*, 1995) membranes, transcription suppression (Tzamarias and Struhl, 1995; Schultz and Carlson, 1987), and *Drosophila* neurogenesis (Lamb *et al.*, 1995). Recently, three members of the human gene family have been isolated (Murthy *et al.*, 1996; Tsukahara *et al.*, 1996; Ohira *et al.*, 1996), although the exact function of these genes is not known.

The short arm of chromosome 1 has been shown to be involved in nonrandom genomic alterations in a variety of different human cancers (Schwab *et al.*, 1996). Observations of structural chromosome rearrangements and loss of heterozygosity (LOH) studies involving chromosome 1p have implied the presence of tumor suppressor genes along its length. In particular, LOH for the 1p31 region has been observed in up to 50% of human breast cancers (Hoggard *et al.*, 1995). Although the human EST sequencing project has generated large numbers of partial gene sequences, only a small proportion of these have so far been assigned to specific chromosomes, and of those that have a chromosomal assignment, many have not been localized to specific chromosome bands. We recently described the mapping of EST markers along the length of human chromosome 1 (Roberts *et al.*, 1996a) using a well-characterized panel of somatic cell hybrids (Roberts *et al.*, 1996b). As a result of this analysis we were able to assign groups of ESTs to five distinct regions of the short arm of chromosome 1. As part of our attempt to clone the translocation breakpoint in a patient with neuroblastoma (Mead and Cowell, 1995), we generated a 6-Mb contig of yeast artificial chromosomes (YACs) in the 1p22 region (Roberts *et al.*, 1998a, b). This contig was shown to link up with the 9-Mb contig described by

INTRODUCTION

The tetratricopeptide repeat (TPR) motif has been identified in a wide variety of genes from many different organisms and with very different functions (Murthy *et al.*, 1996). The TPR motif is a degenerate, 34-amino-acid repeat whose function is mostly unknown, although there are indications that, through formation of helical structures (Das *et al.*, 1998), the motif mediates protein–protein or protein–membrane interactions (Goebl and Yanagida, 1991; Sikorski *et al.*, 1991). Although the first TPR genes, which were identified in bacteria and lower eukaryotes, appeared to be involved

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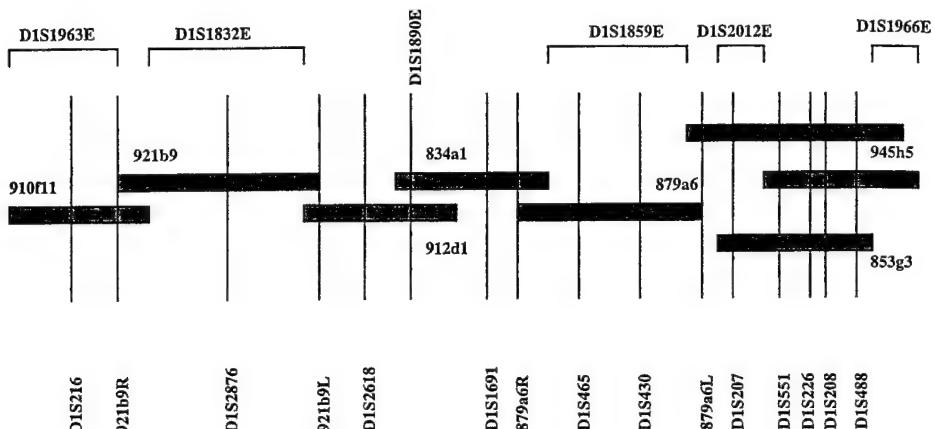


FIG. 1. Physical map of the 1p31 chromosome region frequently deleted in human breast cancer. The extent of the overlapping YACs spanning the minimally deleted region is shown by the horizontal shaded bars. Landmark STS markers from the region are shown below. The location of the six newly mapped ESTs is shown above.

Hoggard *et al.* (1995), which spanned the region of 1p31 frequently shown to undergo LOH in breast tumors. In our original mapping project we assigned 38 novel ESTs, derived from 30 different genes to the 1p21–p31 region. Six of these ESTs mapped to the 6-Mb contig described by Roberts *et al.* (1998a) in 1p22. We have now analyzed the YACs from the contig described by Hoggard *et al.* (1995) with the remaining ESTs from the 1p21–p31 region and sublocalized a further 6 ESTs within this YAC contig. One of these ESTs lay within the minimal region of LOH in breast cancer. Here we describe the isolation and characterization of this gene, *TTC4*, which is a novel member of the TPR family.

MATERIALS AND METHODS

Molecular analyses. cDNA libraries and Northern blots were screened using standard hybridization procedures with 32 P-labeled probes (Sambrook *et al.*, 1989). Northern blots were obtained from Clontech. Filter hybridization was carried out with the ExpressHyb procedure (Clontech).

5' RACE. RACE was carried out with the GIBCO kit as described by the manufacturers using 2 μ g of total RNA from either normal brain or liver (Clontech). Two gene-specific primers (GSP1 and GSP2) were designed. The GSP1, 5'-CTTGCGCTTGCTCCAGTCAA-3', was 927–947 bp of cDNA 487410 from the 5' end. The GSP2, 5'-GTGCAGATCCTGATTGATGC-3', was homologous to bp 1077–1198 of cDNA 487410 from the 5' end. Both of the GSPs were used in combination with the adapter primer (AP1) to amplify the 5' end of the gene. The PCR reaction was performed in 1.5 mM magnesium chloride, 0.2 mM each of the four deoxynucleotide triphosphates, 0.3 mM of each primer, 5 μ l of 10 \times PCR reaction buffer, and 1 U of *Taq* DNA polymerase (GIBCO BRL) in a volume of 50 μ l. RACE products were cloned into pCR2.1 using the TA cloning procedure (Invitrogen) and transformed into JM109 competent cells (Promega). The sequencing analysis of the 5'-RACE PCR product was achieved by using M13 reverse and forward (-20) primers.

PCR and RT-PCR. RNA was isolated using Trizol reagent. Using Superscript II (GIBCO) and random hexamers (Promega), 1–2 μ g of total RNA or 500 ng of mRNA was reverse transcribed. All PCRs were performed using *Taq* polymerase and the buffer supplied by GIBCO with 0.2 mM dNTPs and 0.2 μ M primers. PCR products were analyzed on 3% agarose gels. SSCP was carried out as described by Hogg *et al.* (1992). After the digested RT-PCR products were dena-

tured at 80°C for 5 min, the samples were loaded on the SSCP gel and separated by electrophoresis for 14 h at 6 W.

RESULTS

To map subregionally the 1p22–p32 ESTs identified by Roberts *et al.* (1996a), we selected a minimal set of 8 YACs that spanned 9 Mb in chromosome region 1p31 (Fig. 1). DNA from these YACs was used as the substrate for PCR with the ESTs from 1p22–p31 that did not map to the 1p22 contig described by Roberts *et al.* (1996a). Six of these ESTs were present in the YAC contig (Fig. 1). In the latest release of the human genome map, none of these ESTs has been previously assigned a map location. Of particular interest was the fact that one of these ESTs, D1S1859E, mapped to YAC 879a6, which is central to the overlapping region most commonly undergoing LOH in breast cancer (Hoggard *et al.*, 1995). Because this EST is a potential candidate for the breast cancer gene in 1p31, we characterized it more fully.

The D1S1859E marker was derived from cDNA clone 487410 (ATCC). This clone contains a 2.7-kb insert (data not shown). To facilitate sequencing, we digested this clone with *Hind*III/*Not*I, generating three fragments, 0.4, 0.9, and 1.4 kb long. These fragments were subcloned, and the full 2644-bp sequence was generated. A poly(A) tail was identified at the 3' end, and a 933-bp open reading frame was found between nt 877 and 1809. When cDNA 487410 was used to probe a multiple tissue Northern blot, an approximately 2-kb transcript (Fig. 2) was observed in most tissues. Because this transcript was 600 bp smaller than the cDNA clone insert, the individual subclones were also hybridized to Northern blots. Only the 0.9- and 1.4-kb fragments generated hybridization signals, both of which were 2 kb long. This result indicated that the original 487410 clone was probably chimeric. The 0.9- and 1.4-kb fragments were then used to screen both fetal brain and adult liver cDNA libraries. A 1.7-kb clone (FC4) was isolated from the fetal brain library

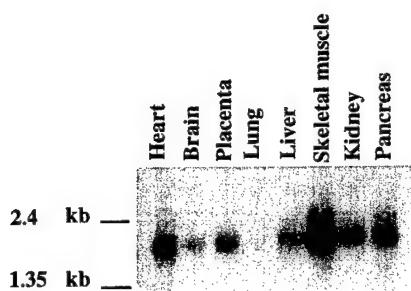


FIG. 2. Northern blot analysis of the distribution of tissue expression of the *TTC4* gene. The multiple tissue blot was probed with cDNA 487410 and detects a 2-kb transcript in all cases. Expression in lung was detectable at low levels.

using the 0.9-kb probe, and a 3.8-kb clone (LC4) was isolated from the fetal liver library using the 1.4-kb probe. These two new clones were sequenced, and, when they were compared with cDNA 487410, it was clear that both of these cDNAs were also chimeric; the 950 bp at the 3' end of FC4 was identical to the 5' end of 487410, whereas the 1784 bp at the 3' end of LC4 was identical to the 3' end of 487410 (Fig. 3). Database homology searches with the 5' end of LC4 revealed homologies with the 3' untranslated region of a gene on chromosome 22. Thus, as a result of this analysis, the 5' end of the gene that maps to chromosome 1p31 was still not accounted for. To find the 5' end we used the Marathon-ready brain cDNA RACE approach. Two gene-specific primers (GSP) were designed from the 5' end of 487410 within the region showing homology with LC4 and FC4 (Fig. 3). Thus, GSP1 annealed to nt 928–947 and GSP2 annealed to nt 1077–1098. These

GSPs were used in combination with the AP1 provided in the RACE procedure. The two resultant bands (Fig. 3), which were 232 and 382 bp long, were subcloned into the pCR 2.1 plasmid vector and then these clones were sequenced. The 232-bp product showed complete homology to the 382-bp fragment at the 5' end (Fig. 3). The sequence at the 3' end of each of these clones was homologous to 487410 (Fig. 3). When the 382-bp fragment was used to probe the same Northern blot used previously, a 2-kb transcript was identified (data not shown). When the 382-bp fragment was incorporated into the 3' sequence of the 487410 cDNA a 2005-bp gene sequence was generated (Genbank Accession No. AF073887). Conceptual translation of this sequence identified a 1071-bp (356 amino acid) open reading frame spanning nt 34–1104 (Fig. 4). This gene contains a tetratricopeptide repeat motif at the 5' end of the gene (Fig. 4). When the amino acid sequence of *TTC4* was analyzed using the coiled-coil prediction program in the ExPASY-Tools (NCBI), a coiled-coil domain that is situated between aa 170 and aa 230 (Fig. 4) was identified.

Homology searches using the SwissProt program revealed 26–41% identity with several genes in the database (Fig. 5). In all cases, the highest homology was within a TPR motif. Amino acid sequence alignment over the TPR domains reveals that *TTC4* has 16% amino acid identity with TPR1 (Murthy *et al.*, 1996), 26% with TPR2 (Murthy *et al.*, 1996), 19% with the transformation-sensitive protein, IEP SSP 3521 (Honore *et al.*, 1992), and 33% with the yeast STI1 protein (Wilson *et al.*, 1994). All of the aligned proteins

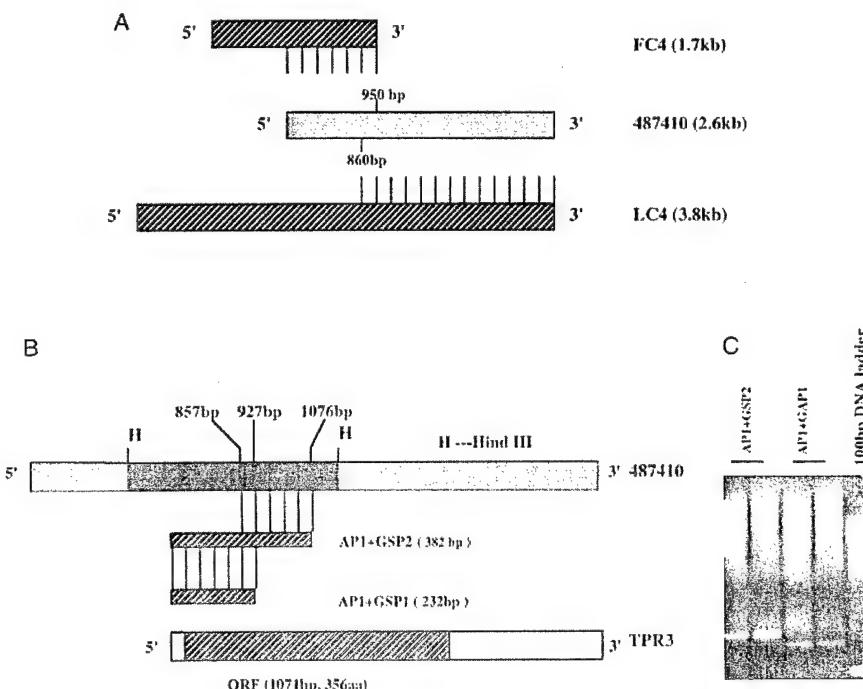


FIG. 3. Construction of the *TTC4* gene sequence. (A) The homologous regions between the original clone, 487410, and the newly isolated clones from fetal brain (FC4) and adult liver (LC4) are shown. (B) The relationship between the two RACE products (C) derived from 487410 that allowed the final gene structure to be established is shown.

FIG. 4. Nucleotide sequence and the deduced amino acid sequence of the *TTC4* cDNA. The four potential TRP repeat motifs are underlined.

contain the degenerate 34-amino-acid motif that defines this structure. TPRs are quite often tandemly arrayed, and it is also common for single and double TPRs to be found separated from the arrays (Lamb *et al.*, 1995). The *TTC4* protein has four separated TPR motifs that are spread throughout the *TTC4* protein. Coiled-coil domains were also found in TPR1, TPR2, IEF SSP 3521, and the yeast STI1 protein (Fig. 4). The location of the coiled-coil domain varies within the proteins. In TPR1 and TPR2 they are found within the TPR domain, whereas in the other genes the coiled coil is located outside the TPR domain toward the carboxy terminal of the gene (Fig. 6). *TTC4* also shows identity with some other proteins in the database such as Lm STI1, serine/threonine protein phosphatase T, and the heat shock protein STI1 (data not shown).

Mutation Analysis in Breast Cancer Cell Lines

Northern blot analysis demonstrates that *TTC4* is expressed in normal breast tissue (data not shown). In a preliminary investigation of whether *TTC4* is mutated in breast cancer cells, we undertook a limited mutation screen in eight breast cancer cell lines (G1C1A, MCF7, MCF7ICI, MDS 435, MDS 453, MDS

468, SKBR3, and T47D) using SSCP. Because we do not have information about the exonic structure of *TTC4*, we analyzed RNA transcripts from these cells. The coding region of *TTC4* (nt 145–945) was amplified using RT-PCR. Because conformation changes are more readily detectable by SSCP in smaller fragments (Hogg *et al.*, 1992), the PCR products were digested with *Bsm*I and *Bgl*II to generate fragment sizes below 300 bp. When the mobilities of the individual fragments on the SSCP gel were compared, we were unable to detect any differences suggesting mutations or polymorphisms. DNA samples from the same eight cell lines were also analyzed by Southern blotting, and no structural rearrangements of this gene were detected. Unfortunately, it was not possible to determine which of these tumors had undergone LOH at 1p31 because the corresponding normal tissue was not available. Given the overall frequency of LOH in breast tumors, however it might be expected on average that 50% of them had experienced LOH in 1p31.

DISCUSSION

The human TTC family of genes previously contained three members, *TTC1* from chromosome region

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26 Y R G G F H E D Q W E K E F E K V P L F M S R A P S E I D P Human TPR3
66 C S A S F E E E - - - P G A D K V E N K S N E D V N S S E L Human TPR1
228 - - - - - - - - - - - - - - - - - - - R M A P D - - - Human TPR2
244 A L K H Y D K A - - - K E L D P T N M T Y I T N O A A V Y F IEF SSP 3521
52 E R K P P D F D D W C K E I D Q H P A F M T E M P T D - - - ySTI1
X R K X F D E D - - - K E X D K V P X F M X E M P X D - - - Consensus

56 R E N P D L A C L Q S I I F D E R S P E E Q - - - - - A Human TPR3
93 D E - E Y - - - L I E L E K N M S D E E K Q K R R E E S Human TPR1
233 H E K A C I A C R N A K A L - - - - - - - - - - - - - Human TPR2
271 E K G D Y I N K C R E C E K A I E V G R E N R E D Y R Q I A IEF SSP 3521
79 - - - - - - - - - - - - - - - - - - - ySTI1
X E - X Y - A C R - L I E L - - E - S - E E - - - - - A Consensus

80 F T Y K D E G N D Y F K E K D Y K K A V I S Y T E G L K K K Human TPR3
117 T R L K E E G N E Q F K K G D Y I E A E S S Y S R A L - - - Human TPR1
247 K A K K E D E G N K A F K E G N Y K L A Y E L Y T E A L G I D Human TPR2
301 K A Y A R I G N S Y F K E E K Y K D A I T H F Y N K S L - - - IEF SSP 3521
79 - - - - - - - - - - - - - - - - - - - ySTI1
K A Y K E E G N X Y F K E G D Y K D A I E S Y T E A L K - - Consensus

110 C A D P D L N A V L Y T N R A A A Q Y Y L G N F R S A L N D Human TPR3
144 - E - - - - - - - - - - - - - - - - - - - M C P - - - - - - - Human TPR1
277 P N N I R T N H A K L Y C N R G T V N S K L R K I H D D A I E D Human TPR2
328 - A E H R T P D V L - - - K K C O O A E K I L K E Q O E R L B A Y IEF SSP 3521
96 S P D R K L N A V L Y F N R A A A Q O K H L G N L R S A I K D ySTI1
- - A D X K L N A V L Y - N R A A A Q X K L G N L R S A L X D Consensus

140 V T A R K L K E C H L K A I I R G A L C H L E L I H F A E Human TPR3
148 - - - - - - - - - - - - - - - - - - - - - - - - - - S C F - Q K E R - - - Human TPR1
307 C T N A V K L D D T Y I K A Y L R R A Q C Y M D T E Q Y E E Human TPR2
355 I N P D L A L E E K N - - - - - - - K G N E C F - Q K G D Y P Q IEF SSP 3521
126 C S M G R K F D P T H L K G V I R G A E C L L E L E Y A K D ySTI1
C T X A R K L D P T H L K A - I R G A E C F L E L E Y X X E Consensus

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FIG. 5. Multiple sequence alignment with a partial amino acid sequence with those from human TPR1, TPR2, IEF SSP 1532, and yeast STI1.

5q32–q33, *TTC2* from chromosome region 17q11–q23, and *TTC3* from chromosome region 21q22 (Murthy *et al.*, 1996; Tsukahara *et al.*, 1996; Ohira *et al.*, 1996). We have now identified the fourth member of this family, *TTC4*, from chromosome region 1p31. *TTC4* contains four repeats of the motif and is apparently ubiquitously expressed, as are all of the other members of the family (Murthy *et al.*, 1996; Tsukahara *et al.*, 1996; Ohira *et al.*, 1996). A role for the TTC gene family in neoplasia has recently been demonstrated in liver tumorigenesis (Isfort *et al.*, 1997). The *Tg737* gene, which contains 10 tetratricopeptide repeats, was identified as the gene that was inactivated by insertional mutation in a transgenic mouse with a phenotype resembling autosomal recessive polycystic kidney disease (Moyer *et al.*, 1994). These mice also showed liver

dysplasia. When Isfort *et al.* (1997) analyzed genetic changes in carcinogen-induced liver tumors in rats, they detected deletions and rearrangements of *Tg737* in 40% of cases. Similar changes were detected in human liver, pancreas, and kidney tumors but not in breast cancers. In cells lacking *Tg737*, overexpression of this gene resulted in a decrease in cell growth *in vitro* and a suppression of the ability of these cells to form tumors in nude mice. More circumstantial evidence that *TTC1* and *TTC2* play a role in tumorigenesis came from a yeast two-hybrid analysis that showed that they interact preferentially, via their tetratricopeptide domains, with a truncated form of the GAP-related domain of the *NF1* gene, which predisposes to the development of neurofibromatosis. Yeast two-hybrid associations do not necessarily reflect intracellular interactions, however, and to date, these interactions have not been confirmed by other means. Another member of the TTC family, p58, has been shown to inhibit the interferon-inducible *PKR* gene (Lee *et al.*, 1994). *PKR* has been suggested to be a tumor suppressor gene, and p58 has oncogenic potential. Thus, although the function of *TTC4* is not known, it might be predicted to have other interesting functions related to tumorigenesis because of its relatedness to other TTC-motif genes. In yeast, for example, some of the cell division cycle genes, which are essential for the progression of the cell cycle, contain TTC repeats (Boguski, 1990; Sikorski *et al.*, 1990). These genes are also homologous to fungal genes that are involved in mitosis (O'Donnell *et al.*, 1991). The human homolog of one of the yeast TPR-containing genes was shown to be upregulated in SV40-transformed human fibroblasts (Honore *et al.*, 1992) compared with their normal counterparts, again possibly suggesting a role in the transformation process. *TTC4* also shares homology to stress-inducible proteins such as STI1 in yeast, a member of a family of genes from various species (Blatch *et al.*, 1997) with a common ancestor. STI1 is important for growth under suboptimal conditions and can either transactivate other genes (Nicolet and Craig, 1989) or, as shown for the homologous *Ski* gene (Toh and Wickner, 1980), regulate RNA synthesis. All of these func-

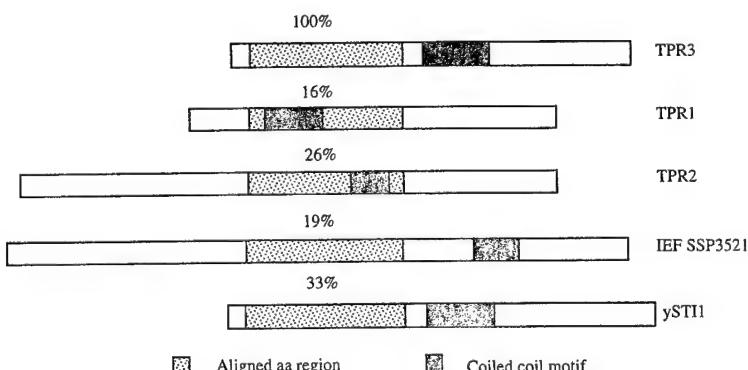


FIG. 6. Diagrammatic representation of the intragenic location and degree of homology of the TPR domains between *TTC4* and other TPR-containing genes. The relative locations of the predicted coiled-coil domains within these genes are also indicated.

tions are potentially relevant to cancer cell growth and survival.

The function of the *TTC3* gene, identified originally as the *TPRD* gene, which was isolated from the Down syndrome (DS) critical region in 21q22 (Tsukahara *et al.*, 1996; Ohira *et al.*, 1996), is also poorly understood, although it is speculated to be responsible for some of the developmental abnormalities seen in DS patients, based on its pattern of expression in the developing mouse (Tsukahara *et al.*, 1998).

The crystal structure of a TPR-containing gene, *PP5*, was recently reported (Das *et al.*, 1998). This study demonstrated that the repeat motif folds into a right-handed superhelical structure, creating a continuous helical groove that would be suitable for the recognition of target proteins. This structure represents a novel mechanism of protein recognition and has the capacity to interact with many proteins and form a multiprotein complex.

The demonstration that *TTC4* maps within the region of LOH in breast cancer makes it a potential candidate for a role in breast tumorigenesis. One unequivocal way of implicating a candidate gene in tumorigenesis is to identify mutations consistently in a specific tumor type. Because LOH is the observation for 1p31 in breast cancer, the prediction is that these would be inactivating mutations, although this is not always the case. The *WT1* gene, for example, is clearly implicated in Wilms tumorigenesis, and, although located within the region of chromosome 11 frequently undergoing LOH in Wilms tumors, mutation analysis can identify mutations in this gene in only a handful of tumors, mostly from patients with specific subtypes of tumors and genetic predispositions (Cowell *et al.*, 1993). At present, we have not established the exon/intron structure of *TTC4*, which would allow mutation analysis at the genomic level. Consequently, in a preliminary screen, we only analyzed RNA from a series of commonly available breast cancer cell lines. The advantages of using these cells are that they are not contaminated by normal cells and that RNA can be prepared from them relatively easily. This is not always true for many of the clinical specimens obtained from breast tumors. Even though no mutations were detected in these experiments, a more extensive screen is still warranted because of the implications of the TTC genes in a variety of cellular functions potentially related to tumorigenesis. Once the structure of the *TTC4* gene has been characterized, an exon by exon analysis would clearly be more informative because this analysis would not be influenced by the consequences of the mutation on gene expression or stability (Kratzke *et al.*, 1994).

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Fine mapping of the PTGFR gene to 1p31 region and mutation analysis in human breast cancer

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Abstract. The 1p31 chromosomal region shows loss of heterozygosity (LOH) in up to 50% of human breast cancer, indicating the presence of a tumor suppressor gene at this location. Many efforts have been made to identify candidate genes responsible for breast cancer on the short arm of chromosome 1. It was shown that prostaglandins have been implicated in the tumorigenesis pathway, perhaps via interactions with their cell surface receptors. The prostaglandin F2 receptor gene (PTGFR) was tentatively mapped to 1p31 adjacent to the region undergoing LOH in human breast cancer. We undertook a mutation study in 34 sporadic human breast tumors using a variant of SSCP, incorporation PCR SSCP (IPS). Several nucleotide variants were detected in different tumors. Here we report the nature of these nucleotide changes and the possible involvement of the PTGFR gene in the etiology of human cancer.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin have been shown to inhibit experimental carcinogenesis in a variety of organs (for review, see ref. 1). All NSAIDs suppress prostaglandin biosynthesis by inhibiting cyclooxygenases, i.e. enzymes catalyzing an essential step in prostanoid formation. In fact, prostaglandins were found to be dramatically elevated in many human and experimental tumors, and prevention of tumor development by NSAIDs was attributed to inhibition of prostaglandin production (1,2). Experimental mouse skin carcinogenesis so far provides the only model in which prostaglandins have been unequivocally shown to act as mediators of regenerative processes and tumorigenesis, especially prostaglandin F2 (3-5). The molecular mechanisms involved in the tumor-promoting activity of prostaglandin F2 (PGF2) are yet to be understood. One candidate pathway through which PGF2 may act is the

interaction with its receptor expressed at the cell surface. FP, also known as PTGFR is the PGF2-specific receptor. PTGFR was tentatively mapped to 1p31 adjacent to the region frequently undergoing LOH in human breast cancer (6,7). The physical location and biological function of the PTGFR gene makes it a good candidate for possible involvement in the etiology of breast cancer. Therefore we undertook a mutation analysis study of PTGFR in 34 human breast tumors.

Materials and methods

YACs, STSs, PCR assays, and physical mapping. YAC contigs of the 1p31 region and marker information has been reported in Hoggard *et al* (6) and is available through the Genome Database (GDB). PCR assays used Taq DNA polymerase (Gibco, BRL) and initial denaturation at 94°C for 4 min followed by 35 cycles of 95°C for 20 sec, 55-65°C for 30 sec, and 72°C for 30 sec. Template genomic DNA varied from 25 to 100 ng per 20-μl reaction.

Incorporation PCR SSCP analysis (IPS). Genomic DNA was isolated from tumor samples as described by Sambrook *et al* (8). IPS was performed as described in Sossey-Alaoui *et al* (9), using primers specific to each exon of PTGFR (Table I).

Results and Discussion

The cloning and characterization of the PTGFR gene (Accession # NM_000959) were described by Kunapuli *et al* (10) and Betz *et al* (7). We confirmed the mapping location of PTGFR to 1p31 using PTGFR gene-specific primers as well as primers derived from STS markers previously proven to map to the breast cancer critical region (6). The map location of the PTGFR gene relative to these STS markers was determined by PCR-based mapping using selected YAC clones from the previously published (6) physical map of 1p31 region (Fig. 1). Furthermore, sequence alignment between PTGFR cDNA and BAC clone RP5-944H6 (Accession # AL136324) clearly identified the fine genomic structure of this gene. It was possible, therefore, to design primer-pairs that could be used in an exon-by-exon mutation analysis. Details of primers used are described in Table I. Because exon 2 is 1,100-bp long and exon 3 is 480-bp long, 5 and 2 separate pairs of primers, which could produce overlapping PCR products, were used to amplify these two exons, respectively. Furthermore, since BAC clone 944H6 is fully sequenced, the exact size of intron 2 could be determined

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Key words: breast cancer, PTGFR, mutation, incorporation PCR SSCP, 1p31

Table I. Primers for PCR amplification of PTGFR exons.

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Annealing temp. (°C)
PTGFREX1	AACGAGTGCGCGGAGGGGAC	CTGGTCCAAACTCAGTGTCAAG	300	65
PTGFREX2-1	GGCCAGATAAAACCCATCCC	AAGATTCCCACTGTCACTGAAGA	302	57
PTGFREX2-2	CCTGCAGCTGCCCTCTTC	CCATGCAGATACCAAAAATACTG	313	60
PTGFREX2-3	CTCATCAATGGAGGCCATAGC	CGCCTGAATTATAGTCTCG	300	53
PTGFREX2-4	CGAAAATTACATCCAAACAT	GAGCTGGATTACCATTCCA	320	53
PTGFREX2-5	ATGCAATCACAGGAATTACAC	CTGAGTGCTGAAATAAATTG	265	55
PTGFREX3-1	AAACAATAGCATCACTCTGTG	CATGCACTCCACAGCATTGA	300	60
PTGFREX3-2	CCGAATGGAACATGGAATC	AGGTATTAACTAACTGAAATATTG	290	55

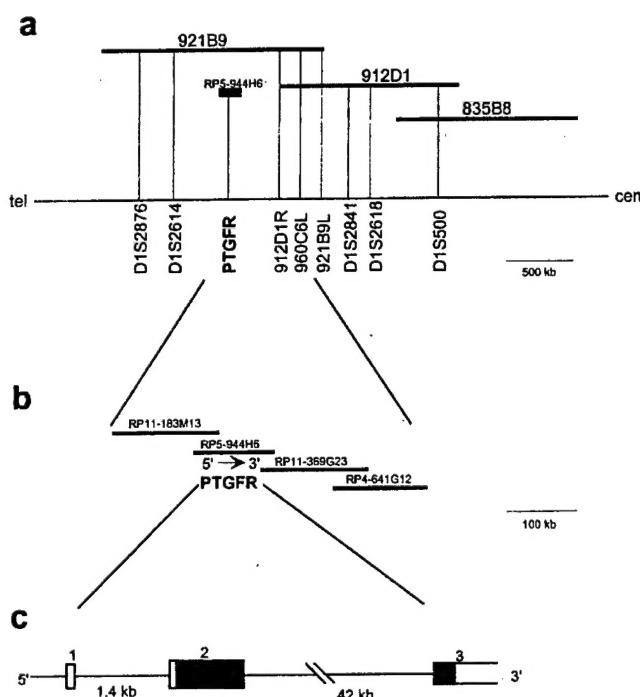


Figure 1. The physical map location of the PTGFR gene. a, Location of the PTGFR gene on the 1p31 physical map. YAC clones are shown in horizontal bars. Positions of STS markers and the PTGFR gene are indicated by thin vertical lines. A detailed STS/YAC map and marker information is given in ref. 5. b, The PTGFR gene maps to BAC clone 944H6 and is transcribed from the telomere to the centromere. A minimum BAC contig was generated using BAC-end sequences and BLAST search against non redundant (nr) and high throughput genomic sequence (htgs) databases. c, Genomic organization of the PTGFR gene.

× (43 kb instead of 6.1 kb as mentioned in ref. 2). Alignment of sequences of BAC clone 944H6 ends with other BAC clones containing STS markers for whom the mapping position is known also determined the transcription direction PTGFR tel-(5'-3')-cen (Fig. 1).

To investigate whether the PTGFR gene is involved in breast cancer, we analyzed DNAs extracted from 34 sporadic

breast tumors using incorporation PCR SSCP. Details of the PCR and SSCP as well as sequencing conditions are described elsewhere (9,11).

We were able to detect four different SSCP changes in several tumors. Fig. 2 shows a representative set of these changes. Subsequent sequencing of the corresponding PCR products identified four single nucleotide changes (Fig. 2), all of which altered the restriction enzyme profile of the corresponding exon, by creating a new or abolishing an existing site of a restriction enzyme. It was then made possible to verify the accuracy of the nucleotide change by using the respective restriction enzyme to digest the corresponding PCR product (data not shown). Two neutral changes were found in exon 2 at positions 63 (C-T, Fig. 2a) and 435 (G-A, Fig. 2b). Neither change affects the amino acid at the corresponding codon (T21T and T145T, respectively). The third nucleotide change, -62 T-C, was detected 62 nucleotides upstream the translation start codon in the 5' untranslated region (UTR) of exon 2 (Fig. 2c). All these three changes were found in only one breast tumor each. Their involvement in breast cancer etiology is yet to be established. The last nucleotide change is a C-T transition, which was found in 26 nucleotides downstream of the acceptor splice site in intron 2 (Fig. 2d). It was clear that this nucleotide change was a polymorphism since it was detected in several tumors in both homozygous and heterozygous states (Fig. 2d).

The PTGFR gene is located in the region of chromosome 1p31 that shows high frequency of LOH in human breast cancer, which makes it a candidate by position. Furthermore, it has been shown that prostaglandins, like PGF2, could be involved in a tumor-promoting process, perhaps via interaction with its specific receptor (PTGFR). Mutations in the PTGFR gene or its specific receptor, PTGFR, could alter such interaction, and therefore lead to tumorigenesis activity. Several nucleotide variants were detected in different regions of the PTGFR gene and in different breast tumors, but none of them appear to be a disease-causing mutation. Two other genes have so far been isolated from the critical breast cancer region in 1p31; LPHH1 (12) and TTC4 (11,13), but based on mutation studies, neither one appears to be involved in breast

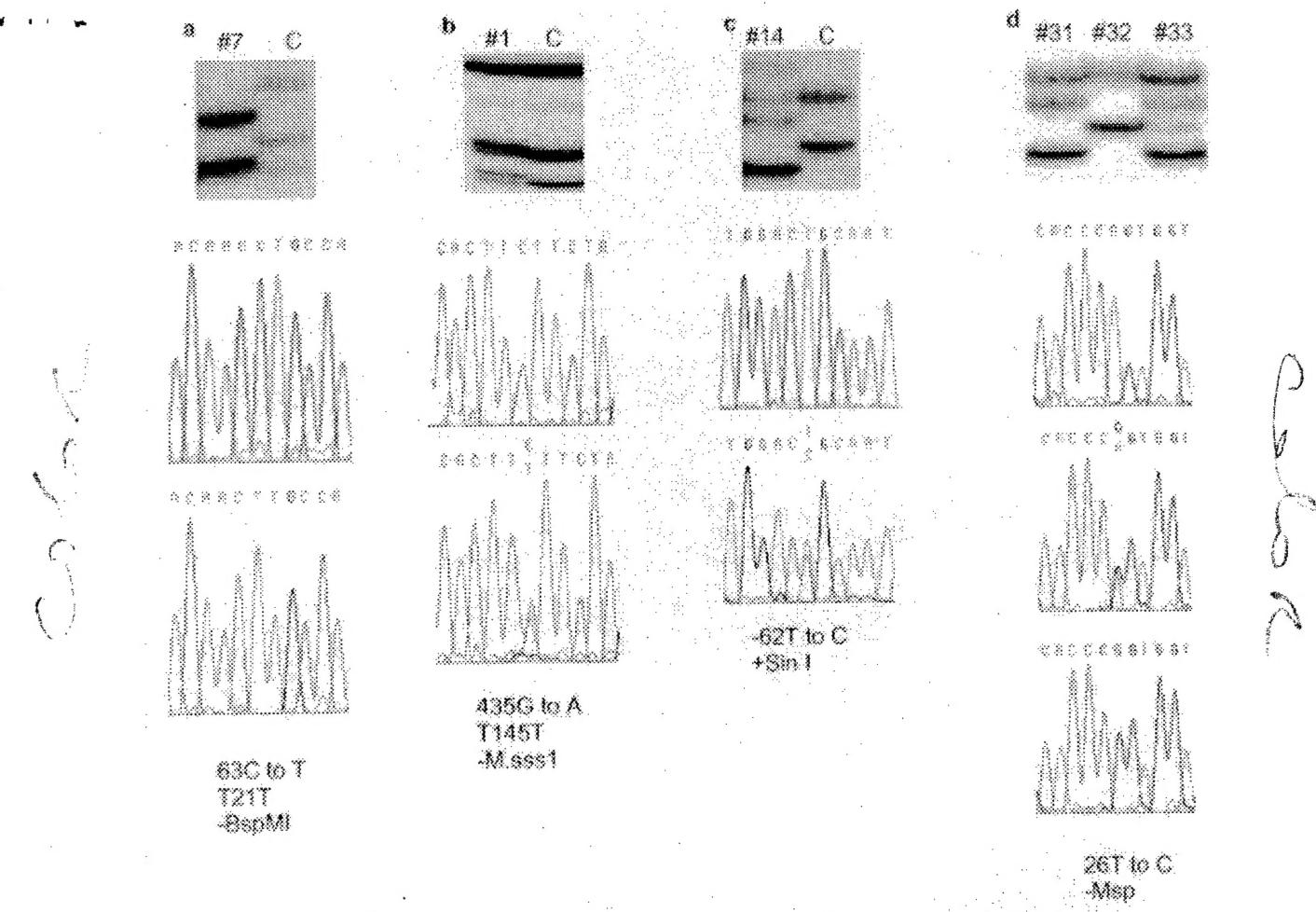


Figure 2. Detection of single nucleotide variations using IPS. Four different SSCP shifts were detected in different breast cancer tumors and are shown in the upper panels of a-d, respectively. Nucleotide sequences corresponding to each SSCP change are shown in the lower panels of a-d, respectively. The nucleotide variation, amino acid change and restriction enzyme site alteration are listed below the sequence traces.

tumorigenesis. Identifying genes from chromosome regions showing LOH is a time-consuming process and, since any gene mapping to the specific region theoretically represents a candidate tumor suppressor gene. Excluding individual genes is an important contribution to the overall characterization of the region. There are, however, a number of other tumors that show LOH involving 1p31, such as melanoma (14-16), neuroblastoma (17) and human male germ cell tumors (18). Although we failed to connect the PTGFR gene to the human breast cancer by mutation screening, the establishment of the PCR and SSCP conditions for this gene will now make it possible for mutation studies in other tumor types showing LOH in the short arm of chromosome 1.

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